Investigations into the effects of a flavonoid- rich berry extract on glucose transport in intestinal epithelial cells and muscle

Cheung, Hoi Man

Awarding institution:
King's College London

Investigations into the effects of a flavonoid-rich berry extract on glucose transport in intestinal epithelial cells and muscle cells

Hoi Man CHEUNG

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Abstract

Flavonoids are a broad collection of polyphenolic compounds ubiquitously found in foods of plant origin. Previous studies suggested that they are beneficial in protecting against many chronic diseases, such as cancers, cardiovascular diseases and neurological disorders. There is also evidence that flavonoids have beneficial effects on modifying glucose absorption and are thus regarded as potential anti-diabetic agents. To address this we investigated the effects of a flavonoid-rich berry extract on glucose transport in intestinal epithelial Caco-2 cells and C2C12 muscle cells.

In Caco-2 cells, acute exposure to berry extract inhibited glucose uptake. Prolonged incubation down-regulated the gene expression of GLUT2 and SGLT1, with a concomitant up-regulation of *let-7a* miRNA. Using bioinformatic tools, *let-7a* is predicted to target the mRNA of GLUT2, potentially down-regulating its expression. In C2C12 myotubes, chronic incubation of berry extract did not have any effect on basal or insulin-stimulated glucose uptake.

Taken together, these data suggested that the acute inhibitory effects of berry flavonoids on glucose transport are specific to intestinal glucose transporters. These compounds may be useful for regulating the rate of glucose absorption from the diet; however, they are less effective modifiers of peripheral glucose transport.

Studies were also carried out on the molecular basis of alcoholic myopathy, using the C2C12 muscle cell model. This provides not only an opportunity to study the pathogenic basis of a common disorder but also to compare putative pathways that may be modulated by myotoxins and myoprotectants. The results of microarray studies showed that alcohol altered the expression of genes related to multiple cellular functions, including insulin signalling, muscle contraction and protein synthesis. On the other hand, microarray studies demonstrated that berry extract was a potent inducer of cellular antioxidant defence system. This indicated that berry extract might act as a dietary strategy to protect cells against ethanol-induced oxidative damage.

For My Parents, Auntie Ruby, Auntie Ping,

Uncle Chris and Ex3E Friends

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3’-/5’-UTR	3’-/5’-untranslated region
ABC	ATP-binding cassette transporter
AGO	Argonaute proteins
AKT	v-akt murine thymoma viral oncogene homolog, also known as protein kinase B, PKB
AMPK	5’ adenosine monophosphate-activated protein kinase
ADP/ AMP/ ATP	Adenosine diphosphate/ adenosine monophosphate/ adenosine triphosphate
ANKRD2	Ankyrin repeat domain-containing protein 2
AP-1	Activator protein 1
AS160	AKT substrate of 160 kDa, also known as TBC1D4
BBMV	Brush border membrane vesicle
BLMV	Basolateral membrane vesicle
C/EBP	CCAAT/ enhancer-binding protein
c-MYC	v-myc myelocytomatosis viral oncogene homolog
CaMKKα/β	Calmodulin-dependent kinase kinase-α/-β
cAMP	Cyclic adenosine monophosphate
$Ca_v1.3$	Calcium channel, voltage-dependent, L type
CCND1	Cyclin D1
DAVID v6.7	The Database for Annotation, Visualisation and Integrated Discovery (DAVID) version 6.7
DMEM	Dulbecco's Modified Eagle's Medium
ECg	Epicatechin gallate
ECM	Extracellular matrix
EGCg	Epigallocatechin gallate
EGC	Epigallocatechin
eIF	Eukaryotic translational initiation factor

GAS1	Growth arrest-specific 1
GEF	GLUT4 enhancer factor
GIP	Glucose-dependent insulinotropic peptide
GLP-1	Glucagon-like peptide 1
GLUT1	Facilitative glucose transporter 1, encoded by the gene, *SLC2A1*, solute carrier family 2, member 1
GLUT2	Facilitative glucose transporter 2, encoded by the gene, *SLC2A2*, solute carrier family 2, member 2
GLUT3	Facilitative glucose transporter 3, encoded by the gene, *SLC2A3*, solute carrier family 2, member 3
GLUT4	Facilitative glucose transporter 4, encoded by the gene, *SLC2A4*, solute carrier family 2, member 4
GLUT5	Facilitative fructose transporter 5, encoded by the gene, *SLC2A5*, solute carrier family 2, member 5
GSH	Glutathione, reduced form
GST	Glutathione-s-transferase
HMOX-1	Heme oxygenase 1
IC_{50}	Half maximal inhibitory concentration
IGF-1	Insulin-like growth factor 1
IR	Insulin receptor
IRS1/2	Insulin receptor substrate 1/2
K_m	Michaelis constant, concentration at which half of maximal transport rate is reached
LKB1	Liver kinase B1
LRR	Leucine-rich repeat
MAPK	Mitogen-activated protein kinase
MAS5.0	Microarray suite version 5.0
MAX	MYC-associated factor X
MEF2	Myocyte enhancer factor 2
miRISC	Micro-ribonucleic acid-induced silencing complex

miRNA	Micro-ribonucleic acid
mRNA	Messenger ribonucleic acid
MXD1	MAX dimerisation protein 1
MYH/ MyHC	Myosin heavy chain
Na^+/K^+-ATPase	Sodium-potassium adenosine triphosphatase, also known as sodium-potassium pump
NADPH	Nicotinamide adenine dinucleotide phosphate
NEK8	NIMA (never in mitosis gene a)-related expressed kinase 8
NMR	Nuclear magnetic resonance spectroscopy
NQO-1	NAD(P)H dehydrogenase, quinone 1
p53	Tumour suppressor protein 53
PCR	Polymerase chain reaction
PI3K	Phosphatidylinositol 3-kinase
PIP_2	Phopshatidylinositol 4,5-bisphosphate
PIP_3	Phopshatidylinositol 3,4,5-triphosphate
PKA	Protein kinase A
PKC	Protein kinase C
PLC	Phospholipase C
Pre-miRNA	Precursor microRNA
Pri-miRNA	Primary microRNA
qRT-PCR	quantitative real time polymerase chain reaction
RBP	RNA binding motif protein
RMA	Robust multiarray average algorithm
ROCK2	Rho-associated coiled-coil containing protein kinase 2
SEM	Standard error of mean
SGLT1	Sodium/glucose co-transporter 1, encoding by the gene, *SLC5A1*, solute carrier family 5, member 1
SLRP	Small leucine-rich proteoglycan
T1R2	Taste receptor, type 1, member 2

T1R3	Taste receptor, type 1, member 3
TBC1D1	TBC1 (tre-2/USP6, BUB2, cdc16) domain family, member 1
TGF-beta	Transforming growth factor-beta
TNNI3	Troponin I, cardiac 3
TPM1A	Tropomyosin 1, alpha
V_{max}	Maximal transport rate

1 Introduction

1.1 Literature review

Glucose is the primary energy source for mammalian cells. In humans, under normal physiological conditions, glucose is the only source of energy for the brain (Nieuwenhuys et al., 1998), though in starvation, it can adapt to utilise ketone bodies. Glucose can be obtained from the diet in various forms of carbohydrates and is also produced in the liver from non-carbohydrate substrates by gluconeogenesis. It is transported in the bloodstream and taken up by body cells, most importantly the muscle cells and adipocytes. The level of glucose in the blood is tightly regulated as a part of the body's metabolic homeostasis (Langfeldt, 1921). In the following section, the mechanisms of glucose transport in human enterocytes and skeletal muscle cells, as well as their regulation will be described.

1.1.1 Glucose transport in human enterocytes

Carbohydrates comprise a major source of energy in the diet. Prior to absorption, carbohydrates are digested into simple monosaccharides, such as glucose, fructose and galactose. Glucose is the principal circulating sugar in blood as a major source of energy and is an important metabolic intermediate. Transport of glucose across the intestinal epithelium has been known for decades to be mediated by glucose transporter proteins, namely sodium/glucose co-transporter 1 (SGLT1, encoded by the *SLC5A1* gene) (Crane, 1962, Hediger et al., 1987) and facilitated glucose transporter 2 (GLUT2, encoded by the *SLC2A2* gene) (Thorens et al., 1988) and to be highly regulated. However, until now, there is still no consensus on the precise mechanisms for the transport of glucose across the brush-border membrane and the basolateral membrane of the intestinal epithelium.

1.1.1.1 Intestinal glucose transport model

In the classical model of intestinal glucose transport (**Figure 1**), glucose is actively transported across the brush-border membrane of intestinal epithelial cells by the sodium/glucose co-transporter 1 (SGLT1) against an 'uphill' gradient (Crane, 1962, Wright, 1998). This active transport process, at the expense of metabolic energy from the hydrolysis of ATP, allows glucose to be absorbed even when the luminal concentration is lower than the circulating blood glucose concentration. Glucose molecules that enter the epithelial cells are then transported out of the cells across the basolateral membrane by the facilitated glucose transporter 2 (GLUT2) (Thorens et al., 1988).

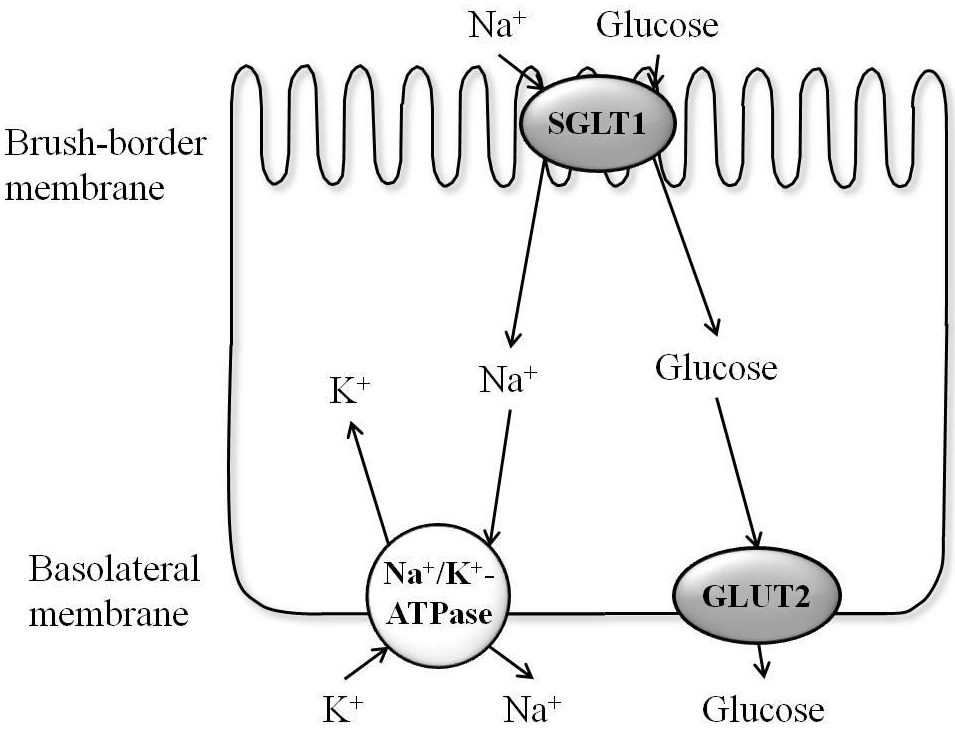

Figure 1. The classical intestinal glucose transport model

Glucose is actively transported across the brush-border membrane of intestinal epithelial cells by SGLT1 against an 'uphill' gradient. The energy for this 'uphill' transport comes from the sodium ion electrochemical potential gradient across the brush-border membrane generated by the basolateral Na^+-K^+-ATPase that pumps the co-transported sodium ions out across the basolateral membrane at the expense of metabolic energy from hydrolysis of ATP. Glucose molecules are then transported out of the cells across the basolateral membrane by the low-affinity high-capacity transporter, GLUT2. Proposed by Wright (1998).

1.1.1.2 Regulation of SGLT1

SGLT1 is one of the most important sugar transporters in the gastrointestinal tract. Defects in the function of SGLT1 give rise to the glucose-galactose malabsorption (Martin et al., 1996), a disorder which is life-threatening to affected individuals unless glucose and galactose are removed from their diets. In the following section, the regulation of SGLT1 in the intestine will be described.

Sorting and expression of SGLT1

The activation of protein kinase A (PKA) has been found to induce a rapid increase in the number of rabbit SGLT1 proteins in the plasma membranes expressed in *Xenopus laevis* oocytes (i.e. within minutes). This led to increased maximal glucose transport rates (Hirsch et al., 1996). These changes were accompanied by an increased area of the plasma membrane, suggesting that regulation occurred by modulating the rate of SGLT1 being inserted into, or retrieved from, the plasma membrane, through exocytosis or endocytosis, respectively (Hirsch et al., 1996, Wright et al., 1997, Khoursandi et al., 2004).

Furthermore, increases in intracellular levels of cyclic AMP (cAMP), a second messenger that activates PKA (Reimann et al., 1971), was also shown to enhance the protein expression of SGLT1 but decrease that of GLUT2 in jejunal enterocytes (Williams and Sharp, 2002). These changes were most prominent in the mid and low villus cells (Williams and Sharp, 2002), which have been suggested to be important zones of enteric adaptation along the villus axis (Debnam et al., 1995).

Direct phosphorylation of SGLT1

More recently, Subramanian et al. (2009) were able to detect an increase in both V_{max} and affinity of glucose transport by rabbit SGLT1 expressed in hamster ovary cells after activation of PKA. In their study, they reported that, in addition to the sorting of SGLT1 between the intracellular space and the plasma membrane, activation of PKA also had a direct effect on SGLT1 by phosphorylation at the PKA consensus site. This resulted in a conformational change in the transporter, altering its functional properties and increasing its transport affinity (Subramanian et al., 2009).

Regulation by dietary sugar and artificial sweetener

Dyer et al. (2003) found that both dietary sugars and artificial sweeteners were able to regulate SGLT1 by altering its mRNA and protein expression (Dyer et al., 2007, Margolskee et al., 2007). This inducible regulation was dependent on the sweet taste receptor, T1R3 and the coupled G-protein, α-gustducin, and acted through the cAMP/PKA signalling cascade (Margolskee et al., 2007). However, these receptors were only present in enteroendocrine cells (Margolskee et al., 2007). The authors proposed that the sweet taste receptors in enteroendocrine cells mediated the release of glucagon-like peptide-1 and glucose-dependent insulinotrophic peptide. These hormones then served as the signal between the sensory enteroendocrine cells and absorptive enterocytes to regulate SGLT1 (Margolskee et al., 2007). They also postulated that *de novo* protein synthesis was required for induction of SGLT1 expression (Dyer et al., 2003), as opposed to other research groups that reported trafficking of SGLT1 (Hirsch et al., 1996, Wright et al., 1997, Subramanian et al., 2009).

1.1.1.3 Regulation of GLUT2

Since 1935, it has been reported that intestinal glucose absorption is comprised of two components (Donhoffer, 1935), the active component mediated by SGLT1 that shows simple kinetics with saturation occurring at around 30 mM glucose, and the apparently non-saturable component that displays linear kinetics from 30 mM to over 100 mM glucose (Debnam and Levin, 1975). Therefore, it is believed that in addition to SGLT1, there is another mechanism by which glucose is transported across the brush-border membrane of intestinal epithelial cells.

The theory of paracellular solvent drag

The first model to explain the diffusive component of intestinal glucose transport was proposed by Pappenheimer and Reiss (1987) as the theory of paracellular solvent drag. It was observed that absorption of glucose through SGLT1 induced the contraction of the perijunctional actomyosin ring and caused the widening of intercellular tight junctions (Madara and Pappenheimer, 1987). The SGLT1-mediated transport of glucose into the cells also provided an osmotic force for solvent flow which permits more glucose molecules to be absorbed through the intercellular space, in a paracellular manner (Pappenheimer and Reiss, 1987).

The theory of negligible diffusive component

Ferraris et al. (1990) argued that the luminal glucose concentration had been over-estimated by other researchers and that the concentration measured in rats on physiological diets in their study ranged from only 0.2 mM to a maximum of 48 mM, which lies within the effective glucose concentration of SGLT1 transport. This supported their view that intestinal glucose absorption could be explained effectively by

the activity of SGLT1 and that the diffusive component of glucose transport was negligible (Ferraris and Diamond, 1997).

The apical GLUT2 trafficking model

The latest model for glucose transport is the rapid insertion of the high-capacity glucose transporter GLUT2 into the brush-border membrane, proposed by Kellett's research group (Helliwell et al., 2000a, Helliwell et al., 2000b, Kellett and Helliwell, 2000) (**Figure 2**). In this model, the level and intrinsic activity of GLUT2 at the brush-border membrane are low when the luminal glucose concentration is low, while SGLT1 acts as the major glucose transporter on the brush-border membrane at this stage (Kellett and Helliwell, 2000). After a meal, when the local concentration of glucose in the intestinal lumen increases, intense SGLT1-mediated transport of glucose across the brush-border membrane activates protein kinase C βII (PKCβII). This in turn increases the intrinsic activity of GLUT2 already at the brush-border membrane, and more importantly, promotes the trafficking of additional GLUT2 to the membrane (Kellett and Helliwell, 2000). This notion was supported by studies which showed that the increase in both the GLUT2 protein expression and the GLUT2-mediated glucose uptake in response to a glucose bolus were blunted in $SGLT1^{-/-}$ mice (Gorboulev et al., 2012). This rapid translocation of GLUT2 towards the brush-border membrane provides a cooperative mechanism by which the absorptive capacity of glucose can be rapidly and precisely adjusted according to dietary intake immediately after a meal.

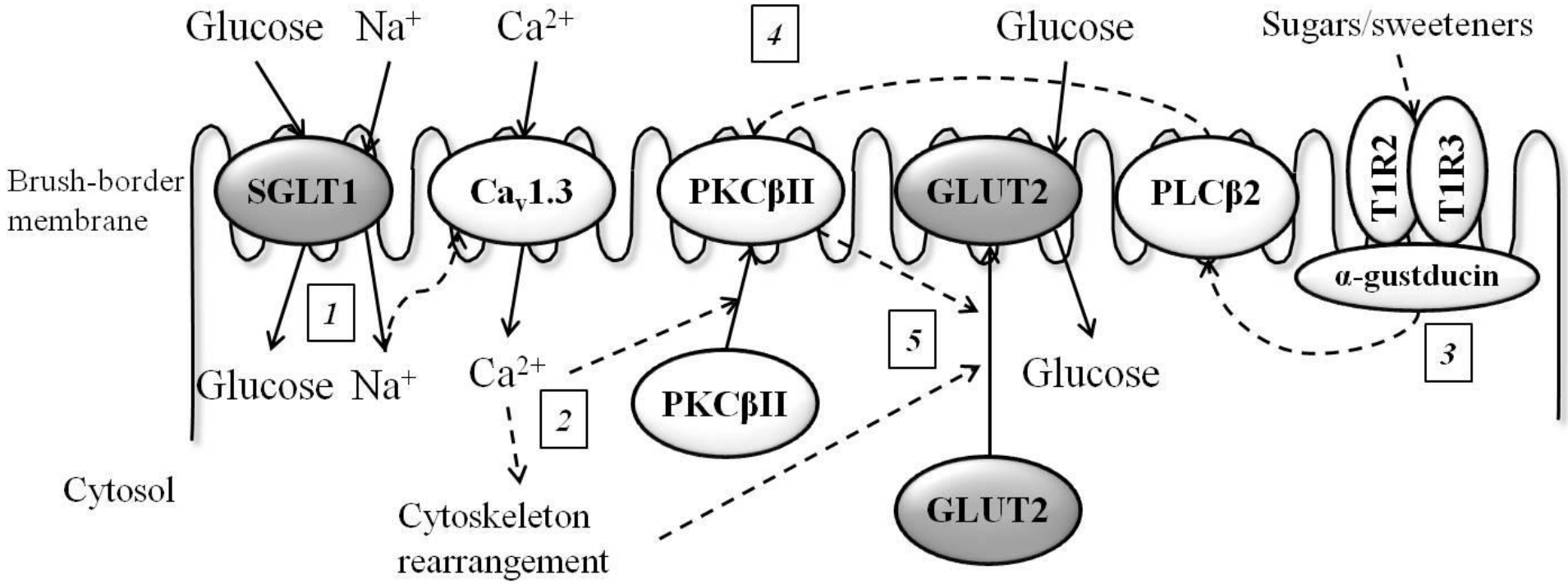

Figure 2. The apical GLUT2 trafficking model

1) Intense Na^+-coupled transport of glucose depolarises the brush-border membrane, triggering an influx of extracellular Ca^{2+} ions through the $Ca_v1.3$ calcium channel; ***2)*** increased level of cytosolic Ca^{2+} ions induces a re-arrangement of cytoskeleton and contraction of the terminal web, as well as the translocation of PKCβII to the brush-border membrane; ***3)*** stimulation of the T1R2+T1R3 taste receptors by sugars or artificial sweeteners activates PLCβ2 through α-gustducin; ***4)*** PLCβ2 activates PKCβII at the brush-border membrane; ***5)*** fully activated PKCβII, together with the re-arrangement of cytoskeleton, promotes trafficking of GLUT2 to the brush-border membrane. Drawing was based on information from various sources: Kellett and Helliwell (2000), Morgan et al. (2007), Mace et al. (2009).

Calcium-dependent regulatory pathway

Following initial findings, Kellett's research group has further investigated the molecular mechanisms by which the insertion of GLUT2 proteins into the brush-border membrane is regulated (Mace et al., 2007a, Mace et al., 2007b, Morgan et al., 2007, Mace et al., 2009) (**Figure 2**). They observed that apical GLUT2 insertion requires an increase in cytosolic Ca^{2+} concentration (Mace et al., 2007b, Morgan et al., 2007). When luminal glucose level rises to a concentration of 20 mM, Na^{+}-coupled transport of glucose depolarises the brush-border membrane, triggering an influx of extracellular Ca^{2+} ions through the voltage-dependent L-type $Ca_v1.3$ calcium channel (Morgan et al., 2003). The increased level of cytosolic Ca^{2+} ions induces a re-arrangement of the cytoskeleton and contraction of the terminal web, thus providing the prerequisite for the trafficking of GLUT2 to the apical membrane (Mace et al., 2007b). The increased intracellular Ca^{2+} level also promotes the translocation of phosphorylated, but not yet fully activated, PKCβII, from the cytosol to the brush-border membrane (Newton, 1997), which requires activation by additional taste receptor signals to finally induce the GLUT2 translocation process (Mace et al., 2007a, Mace et al., 2009).

Sweet taste receptor signalling pathway

An increase in intracellular Ca^{2+} level is a prerequisite for, but is not sufficient to trigger, GLUT2 translocation. A second signal from the sweet taste receptor signalling pathway is also necessary (Mace et al., 2007a, Mace et al., 2009). Sugars as well as non-metabolisable artificial sweeteners contribute to the migration of GLUT2 into the brush-border membrane (Mace et al., 2007a). Sugars at high concentrations (up to 100 mM) or artificial sweeteners at low concentrations (< 5 mM) (Li et al., 2002) activate the sweet taste receptor heterodimer T1R2+T1R3 located at the brush-border membrane. Stimulation of T1R2+T1R3 activates its coupled G-protein α-gustducin which

subsequently completes the activation of PKCβII to promote the translocation of GLUT2 to the apical membrane within minutes (Adler et al., 2000; Mace et al., 2009). However, a recent study in humans failed to demonstrate any effects of intraduodenal administration of sucralose, an artificial sweetener, on the rate of glucose absorption from the lumen of the small intestine (Ma et al., 2010). This argued against the involvement of sweet taste receptors in the regulation of intestinal glucose absorption.

Hormonal regulation

In addition to the rapid adjustment of glucose absorptive capacity according to changes in dietary intake, intestinal glucose transport was also found to be under hormonal regulation (Helliwell et al., 2000a, Shepherd et al., 2004, Tobin et al., 2008). The pancreas secretes insulin in response to increased blood glucose level. The level of insulin release is further enhanced by the secretion of incretins (glucagon-like peptide 1, GLP-1 and glucose-dependent insulinotropic peptide, GIP) by enteroendocrine cells in response to exposure of the gut to nutrients (Baggio and Drucker, 2007). Insulin reaches the enterocytes and promotes the internalisation of GLUT2 from the brush-border membranes to the intracellular membrane stores (Tobin et al., 2008), possibly by acting through the extracellular signal-regulated kinase, p38 mitogen-activated kinase and phosphatidylinositol 3-kinase intracellular signalling pathways (Helliwell et al., 2000a). This returns the GLUT2 transporter to its configuration during low luminal glucose levels and limits the transepithelial transport of glucose.

1.1.1.4 Alternative pathway for glucose transport across the basolateral membrane

It is generally believed that after glucose molecules enter the intestinal epithelial cells, the majority of the glucose, if not all, is then released into the interstitial space across

the basolateral membrane by facilitated diffusion via GLUT2. In this regard, Stümpel et al. (2001) have demonstrated that glucose might be able to exit the enterocytes by another transport mechanism independent of GLUT2. They reported normal kinetics of intestinal glucose absorption in GLUT2-null mice, whereas the intestinal glucose transport was inhibited dose-dependently by a glucose-6-phosphate translocase inhibitor. Furthermore, they found that the GLUT2-null mice were unable to transport 3-*O*-methylglucose, a non-metabolisable glucose analogue that cannot be phosphorylated (Stümpel et al., 2001). These studies suggested that there might be an alternative pathway for glucose exit across the basolateral membrane, which requires glucose phosphorylation, transport of the glucose-6-phosphate into endoplasmic reticulum, and, after dephosphorylation, release out of the cells by means of vesicle transport (Stümpel et al., 2001). A human study on a GLUT2-deficient patient also showed that GLUT2 was not required for intestinal transepithelial glucose transport (Santer et al., 2003).

1.1.1.5 GLUT1, GLUT3 and GLUT5

In addition to the two major glucose transporters, GLUT2 and SGLT1, other hexose transporters are also present in the gastrointestinal tract. These include GLUT1 (erythrocyte-type), GLUT3 (brain-type) and GLUT5 (specific fructose transporter).

GLUT1 is highly expressed in all fetal tissues and is also widely expressed in various adult tissues (Olson and Pessin, 1996). Its ubiquitous distribution and transport kinetics (K_m close to normal blood glucose level, i.e. around 5 mM) suggest that GLUT1 plays an important role in maintaining low level of basal glucose uptake required to sustain respiration in all cells. Specifically, expression of GLUT1 is low in the small intestine and is predominantly located intracellularly and on the basal side of the intestinal epithelial cells (Boyer et al., 1996, Yoshikawa et al., 2011).

GLUT3 is a high-affinity and high-capacity glucose transporter. It is highly expressed in tissues with high energy demand, such as brain, neurons and sperms (Simpson et al., 2008). Abundance of GLUT3 mRNA was found to be very low in normal small intestine tissue (Yano et al., 1991) but its expression was elevated in various cancers (Yamamoto et al., 1990).

GLUT5 is a transporter with high stereospecificity for fructose. Along the gastrointestinal tract, mRNA of GLUT5 was detected predominantly from the proximal to middle parts of the small intestine (Yoshikawa et al., 2011). Within the enterocyte, GLUT5 transporters are mainly present on the brush-border membrane, but has also been detected in the basolateral membrane (Blakemore et al., 1995). Regulation of GLUT5 expression by sugars is specific to its substrate, fructose (or sucrose which undergoes hydrolysis to fructose and glucose) (Miyamoto et al., 1993). It is believed that *de novo* GLUT5 mRNA and protein synthesis is required for the observed increases in fructose transport after fructose feeding (Lan and Ronaldo, 2001). On the other hand, cAMP has also been demonstrated to activate transcription of GLUT5 by interacting with the *cis*-regulatory sequences in the GLUT5 promoter region, resulting in increases in both mRNA, protein levels of GLUT5 and fructose transport activity in Caco-2 cells (Mahraoui et al., 1994b).

1.1.2 Glucose transport in skeletal muscle cells

Skeletal muscle contributes approximately 40% of body weight and is the principal site of postprandial glucose disposal (Zurlo et al., 1990). It has been estimated that under insulin-stimulated conditions, skeletal muscle accounts for approximately 75-80% of whole body glucose uptake following glucose infusion (Defronzo et al., 1985).

Glucose uptake into skeletal muscle cells is primarily mediated by the insulin-responsive glucose transporter type 4 (GLUT4), although GLUT1, GLUT5 and GLUT12 are also present (Stuart et al., 2000, Stuart et al., 2006). Being the major glucose transporter in skeletal muscle, as well as in adipose tissues, GLUT4 is indispensable for normal whole-body glucose homeostasis.

1.1.2.1 Regulation of GLUT4

In order to tightly regulate blood glucose concentration under various physiological conditions, the distribution of GLUT4 between the cell surface and intracellular sites is rapidly regulated in response to specific stimuli such as insulin and muscle contraction independent of changes in transcription and translation. For instance, in non-stimulated cells, the majority of GLUT4 resides within intracellular membrane compartments with less than 5% of the total GLUT4 pool localised at the plasma membrane. However, following insulin stimulation, up to 50% of the total GLUT4 pool redistributes from its intracellular locations to the plasma membrane (Leney and Tavare, 2009). In addition to acute regulation of its distribution through translocation to the cell surface, GLUT4 is also subjected to transcriptional regulation. One example is the increase in GLUT4 expression in response to exercise and physical training (Maclean et al., 2002).

GLUT4 translocation

GLUT4 transporters are capable of trafficking between the plasma membrane and intracellular membrane compartments in response to various stimuli such as insulin, contraction, depolarisation and energy deprivation. However, translocation of GLUT4 is mediated by distinct mechanisms under different stimulations. The two most studied signal transduction pathways involved in GLUT4 trafficking in muscle are the insulin signalling and AMPK pathways.

Insulin signalling pathway

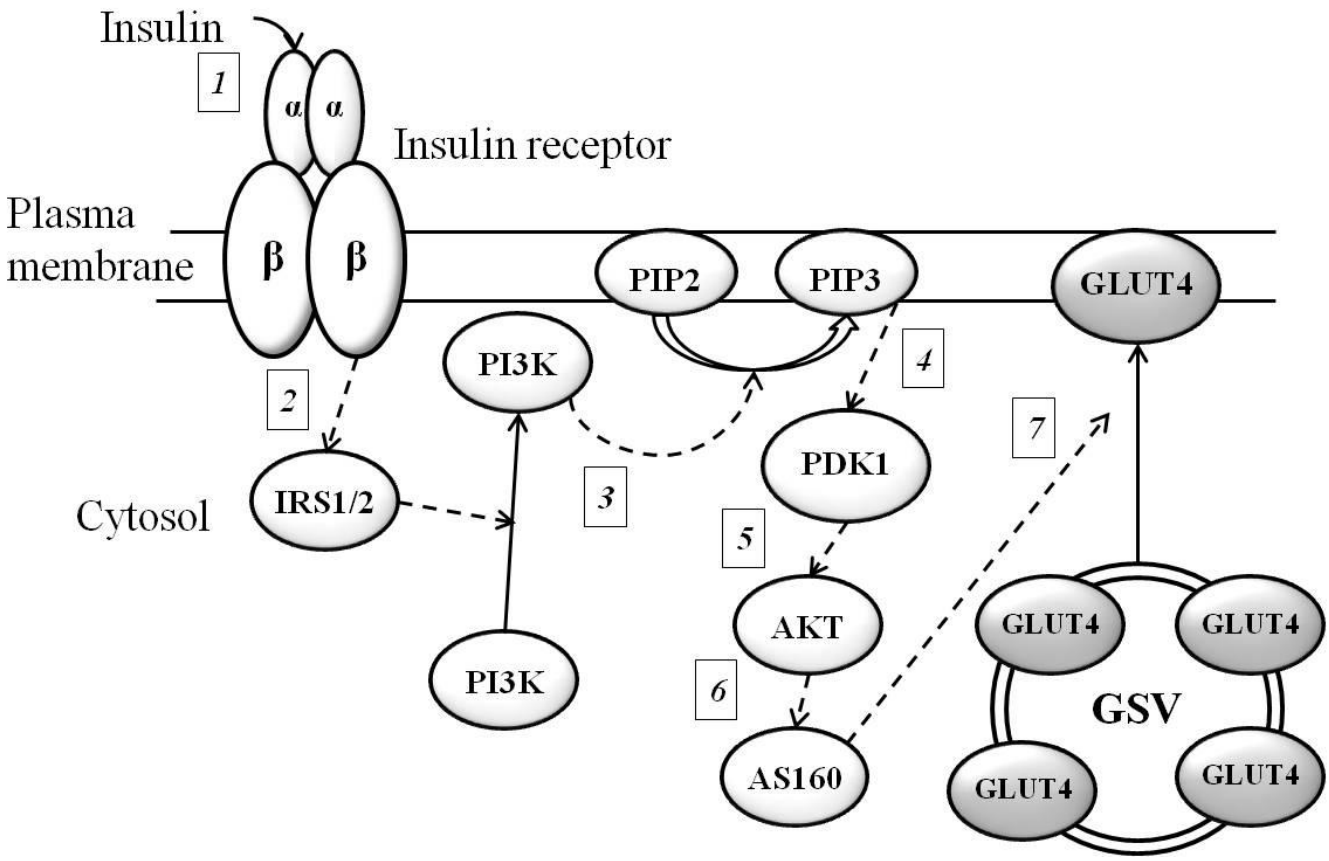

Figure 3. The insulin signalling pathway

1) Binding of insulin induces autophosphorylation of IR tyrosine kinase; ***2)*** activated IR phosphorylates IRS1/2 and subsequently ***3)*** leads to recruitment of PI3K to the plasma membrane; ***4)*** PI3K converts the membrane-bound PIP_2 to PIP_3 which activates PDK1, which ***5)*** further activates AKT; ***6)*** downstream targets of AKT that leads to stimulation of glucose uptake include, the Rab GTPase-activating protein, AS160; ***7)*** phosphorylation of AS160 suppresses its inhibitory effect on Rab proteins, thus promoting the transclocation of GLUT-4 storage vesicles (GSVs) to the plasma membrane. Drawing was based on information from various sources: Kahn et al. (1985), Sanchezmargalet et al. (1994), Berwick et al. (2004), Larance et al. (2005), Geraghty et al. (2007), Leney and Tavare (2009).

Upon binding of insulin to the insulin receptor (IR) tyrosine kinase, it undergoes autophosphorylation (**Figure 3**). This leads to phosphorylation of insulin receptor substrate proteins (IRS1/2) and the recruitment of phosphatidylinositol 3-kinase (PI3K) to the plasma membrane, which subsequently catalyses the conversion of the membrane bound phosphatidylinositol 4,5-bisphosphate (PIP_2) to phosphatidylinositol 3,4,5-

triphosphate (PIP_3). PIP_3 in turn recruits and activates the serine/threonine kinase phosphoinositide-dependent kinase 1 (PDK1), which further activates another protein kinase, AKT. Downstream targets of AKT include the Rab GTPase-activating proteins, TBC1 (Tre-2/USP6, BUB2, cdc16) domain family member 1 (TBC1D1) and AKT substrate of 160 kDa (AS160, also known as TBC1D4) and FYVE domain-containing PI3P 5-kinase (PIKfyve) (Berwick et al., 2004, Chen et al., 2008, Peck et al., 2009).

AMPK pathway

Muscle contraction and insulin stimulation are believed to target separate pools of intracellular GLUT4-containing membranes. Moreover, there is convincing evidence demonstrating that muscle contraction-induced GLUT4 translocation is mediated by signalling mechanism different from the insulin signalling pathway, namely the AMPK pathway. Two cellular responses, increases in either AMP/ATP or ADP/ATP ratios, or an increase in intracellular Ca^{2+} concentrations, during muscle contraction were demonstrated to trigger the activation of the 5' adenosine monophosphate-activated protein kinase (AMPK) (Hayashi et al., 1998) (**Figure 4**).

Changes in energy status

As an energy sensor, AMPK can be activated by an increase in energy demand as indicated by a rise in either AMP/ATP or ADP/ATP ratios during muscle contraction. Binding of AMP to AMPK causes allosteric activation of the kinase (Suter et al., 2006). In addition, the upstream kinase, LKB1, constitutively phosphorylates AMPK but in the presence of high ATP concentration, it is constantly dephosphorylated by protein phosphatases. AMP or ADP binding to AMPK induces a conformational change that reduces the accessibility of phosphatases to the kinase, thus preventing its dephosphorylation (Davies et al., 1995, Suter et al., 2006).

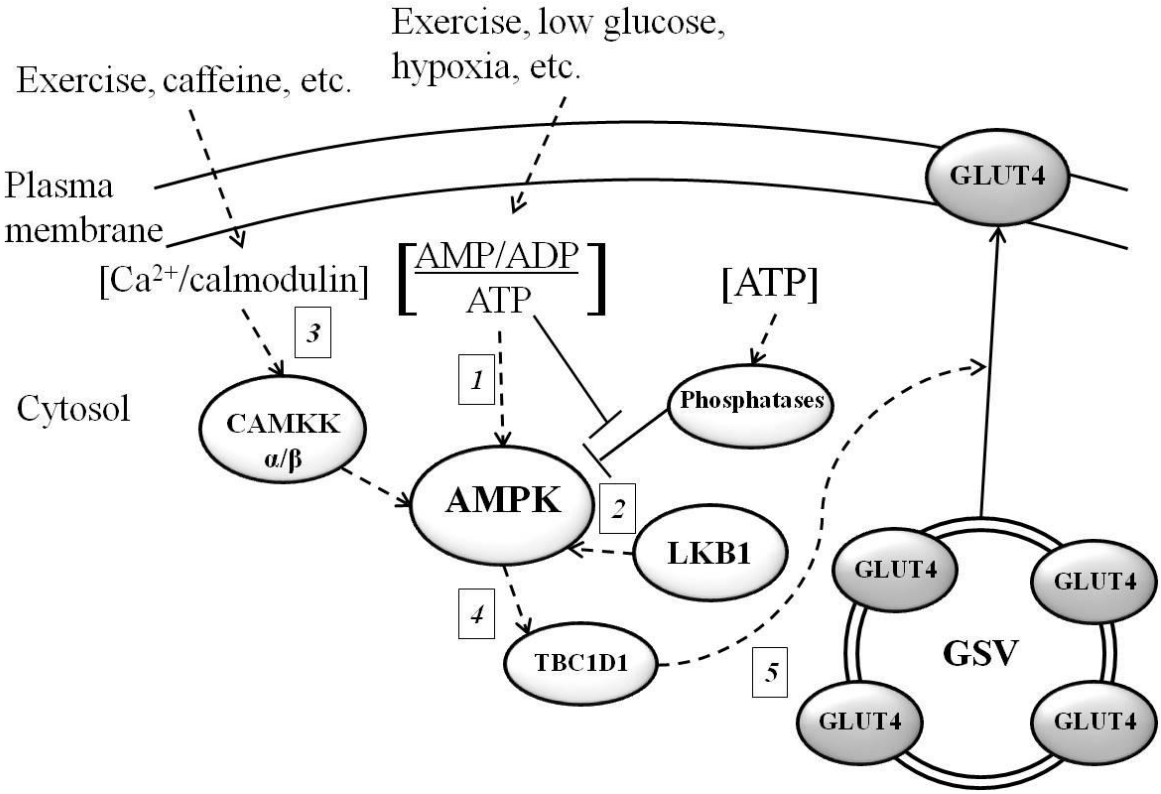

Figure 4. The AMPK signalling pathway

1) Increases in either AMP/ATP or ADP/ATP ratios triggered by energy-demanding conditions, such as exercises, activates AMPK; ***2)*** the upstream kinase, LKB1, constitutively phosphorylates AMPK but it is constantly dephosphorylated by protein phosphatases when ATP level is high, in contrast, AMP or ADP binding to AMPK induces a conformational change that reduces the accessibility of phosphatases to the kinase, thus preventing its dephosphorylation; ***3)*** an increase in cytosolic Ca^{2+} concentration also activates AMPK through another upstream kinase, CAMKKα/β; ***4)*** AMPK phosphorylates the Rab GTPase-activating protein, TBC1D1; ***5***) phosphorylation of TBC1D1 suppresses its inhibitory effect on Rab proteins, thus promoting the transclocation of GLUT-4 storage vesicles (GSVs) to the plasma membrane. Drawing was based on information from various sources: Davies et al. (1995), Hurley et al. (2005), Suter et al. (2006), Abbott et al. (2009), Witczak et al. (2010).

Intracellular calcium concentrations

As mentioned above, increased intracellular Ca^{2+} concentrations also trigger phosphorylation of AMPK. This occurs through binding of Ca^{2+}/calmodulin to, and subsequent activation of, the upstream kinases, calmodulin-dependent kinase kinase-α and -β (CaMKKα and β) (Hurley et al., 2005, Witczak et al., 2010).

Transcriptional regulation of GLUT4

In addition to translocation of the GLUT4 transporter between intracellular regions and the plasma membrane, expression of the GLUT4 gene is also subjected to homeostatic regulation. One example is the ability of exercise to induce a transient increase in GLUT4 gene transcription in skeletal muscle (Neufer and Dohm, 1993, Kraniou et al., 2000).

Cis-DNA sequences in the GLUT4 promoter region bind the transcription factors, myocyte enhancer factor 2 (MEF2) and GLUT4 enhancer factor (GEF) (Maclean et al., 2002, Holmes et al., 2005). Regulation of the activities of these transcription factors and their binding to the GLUT4 promoter has been suggested to signal through AMPK, CaMK and p38 MAPK (Maclean et al., 2002, Al-Khalili et al., 2004, Murgia et al., 2009, Ojuka et al., 2012).

1.1.3 Glucose transport and type 2 diabetes

Type 2 diabetes is a complex metabolic disorder. The major defect of type 2 diabetes is insulin resistance- a pathophysiological condition in which cells fail to respond to the normal action of insulin for glucose homeostasis, most importantly for glucose disposal in skeletal muscle and suppression of endogenous glucose production in the liver (Dinneen et al., 1992). Physiological and molecular changes relating to aberrations in glucose transport have been reported in type 2 diabetes.

1.1.3.1 Gastrointestinal tract

The gastrointestinal tract is the first site where food from the external environment is digested and the resultant nutrients are taken up into cells. Alterations in the normal

digestion and absorption processes have been reported in type 2 diabetic patients, as described below.

Expression and sub-cellular localisation of intestinal glucose transporters

Enhanced intestinal glucose absorption has been reported in diabetic rats (Fedorak et al., 1987). Although this can be partly accounted for by generalised mucosal hypertrophy in diabetic conditions (Fedorak et al., 1987), studies have attributed the enhanced glucose absorption to the increased expression of the glucose transporters GLUT2 and SGLT1 (Fedorak et al., 1987, Miyamoto et al., 1991, Burant et al., 1994). However, these aforementioned studies were performed using streptozotocin-induced diabetic rats, which are considered as an animal model for type 1 diabetes. Later investigations have been made using a more suitable model, namely obese type 2 diabetic Otsuka Long-Evans Tokushima Fatty (OLETF) rat (Kawano et al., 1994). Increased glucose absorption was also observed in this model. This was found to be the result of an increased level of SGLT1 protein concomitant with intestinal hypertrophy in the OLETF rats (Fujita et al., 1998).

In humans, enhanced intestinal glucose transport was observed in brush-border membrane vesicles isolated from type 2 diabetic patients in comparison to healthy subjects and this was explained by the increase in the expressions of GLUT2 and SGLT1 (Dyer et al., 2002). In accordance with the above finding, apical GLUT2 in jejunal enterocytes was observed in most morbidly obese human subjects (76%) in the fasting state, in contrast to its basolateral-only location in lean subjects (Ait-Omar et al., 2011).

In addition, abundance of the fructose transporter GLUT5, and abundances as well as activities of sucrase and lactase were also up-regulated in the duodenum of type 2 diabetic patients (Dyer et al., 2002).

Secretion and insulinotropic action of incretin hormones

Incretins, mainly referring to glucagon-like peptide-1 (GLP-1) and glucose-dependent insulinotropic polypeptide (GIP), are peptide hormones secreted by the gut endocrine cells. Both GLP-1 and GIP are able to induce the release of insulin from pancreatic β-cells. Mixed results have been published on the level of GIP in type 2 diabetic patients compared to non-diabetic subjects, i.e. either increases, no change or decreases in GIP secretion were observed. In comparison to GIP, reports of impairments in GLP-1 secretion in response to oral glucose or meal ingestion in type 2 diabetes are more consistent (summarised in **Table 1**).

Table 1. Changes of secretion and responsiveness of GIP and GLP-1 in type 2 diabetes

		Changes in type 2 diabetes	References
Secretion	**GIP**	Basal ↑; postprandial ↑	Coxe et al. (1981)
		Postprandial ↓, NC, ↑	Creutzfeldt et al. (1983)
		Postprandial ↓	Service et al. (1984)
		Basal ↑/NC; postprandial ↑/NC	Lardinois et al. (1985)
		Postprandial ↑	Schauder et al. (1977); Ross et al. (1977); Takemura et al. (1981); Crockett et al. (1976)
		Basal ↑; postprandial ↓	Elahi et al. (1984)
		Basal NC; postprandial NC	Vilsboll et al. (2001, 2003)
	GLP-1	Postprandial ↓	Vilsboll et al. (2001, 2003); Vaag et al. (1996); Toft-Nielsen et al. (2001)
Responsiveness	**GIP**	Postprandial ↓	Elahi et al. (1994); Nauck et al. (1993); Jones et al. (1987); Krarup et al. (1987)
	GLP-1	Postprandial NC	Elahi et al. (1994); Nauck et al. (1993)

GIP: glucose-dependent insulinotropic polypeptide; GLP-1: glucagon-like peptide-1. ↑: increase; ↓: decrease; NC: no change. Materials compiled from various sources as indicated.

In addition to the alterations in the amounts of incretin secreted, changes in the responsiveness of pancreatic β-cells to these incretins to release insulin in type 2 diabetes also occur. Several research groups have reported marked attenuation in the insulinotropic action of GIP in type 2 diabetic patients. On the other hand, the insulinotropic action as well as the glucagon lowering property of GLP-1 were shown to be preserved in type 2 diabetic patients, in spite of a slight reduction of the incremental response of insulin secretion compared to healthy volunteers (summarised in **Table 1**).

1.1.3.2 Skeletal muscle

In the postprandial state, when blood glucose rises and insulin is released in response to the increased blood glucose level, skeletal muscle accounts for about 75-80% of whole body glucose disposal (Defronzo et al., 1985). In skeletal muscle of type 2 diabetic patients, glucose uptake stimulated by insulin is reduced by about 50% compared to healthy controls (Defronzo et al., 1985, Andreasson et al., 1991, Bonadonna et al., 1996). Researchers revealed that this defect was related to impairments along the insulin receptor signalling cascade.

Insulin receptor (IR)

The binding of insulin to the insulin receptor (IR) on the cell surface and the subsequent auto-phosphorylation of the tyrosine kinase are the first recognised events that initiate glucose transport in muscle cells (Kahn, 1985). In skeletal muscle of type 2 diabetic patients, binding efficiency and protein level of IR were generally believed to be unaltered (Arner et al., 1987, Krook et al., 2000). However, some studies demonstrated that reduced muscle IR tyrosine kinase activity occurs in type 2 diabetic patients (Arner

et al., 1987, Maegawa et al., 1991, Nolan et al., 1994), though others observed no significant differences (Klein et al., 1995, Krook et al., 2000) (summarised in **Table 2**).

Insulin receptor substrate-1 (IRS-1) and phosphatidylinositol 3-kinase (PI3K)

Activation of IR triggers phosphorylation on tyrosine residues of downstream targets, including insulin receptor substrate-1 (IRS-1), the predominant downstream signalling molecule of IR in human skeletal muscle. Extensive investigations have been carried out into the impairment of IRS-1 phosphorylation in type 2 diabetes. Reports of a reduction in tyrosine phosphorylation of IRS-1 were consistent (Goodyear et al., 1995, Bjornholm et al., 1997, Krook et al., 2000, Pratipanawatr et al., 2001). In addition, it has been shown that in cultured skeletal muscle cells from type 2 diabetic patients, there was an increased basal serine phosphorylation of IRS-1, which reduced the ability of IRS-1 to be tyrosine-phosphorylated by IR, resulting in impaired insulin signalling (Aguirre et al., 2002). This provides evidence for the pathogenesis of type 2 diabetes, as serine phosphorylation of IRS-1 can be induced by fatty acids (Yu et al., 2002), chronic insulin exposure (hyperinsulinemia) (Pederson et al., 2001, Rui et al., 2001, Zhande et al., 2002), tumor necrosis factor-α (Rui et al., 2001, Plomgaard et al., 2005), to name a few processes. In contrast, adiponectin, an adipokine that shows reduced levels in obesity (Weyer et al., 2001, Gil-Campos et al., 2004) and type 2 diabetes (Hotta et al., 2000, Weyer et al., 2001), was found to sensitise insulin signalling by inhibiting serine phosphorylation of IRS-1 (Wang et al., 2007).

Phosphatidylinositol 3-kinase (PI3K) is an insulin signal transducer which is activated via interaction with IRSs. In accordance with the reduction in IRS-1 tyrosine phosphorylation in type 2 diabetes, impairment in kinase activity of PI3K also occurs

(Goodyear et al., 1995, Bjornholm et al., 1997, Kim et al., 1999, Krook et al., 2000) (summarised in **Table 2**).

Table 2. Changes along the insulin signalling pathway in muscle in type 2 diabetes

		Change	References
IR	Protein level	NC	Arner et al. (1987); Krook et al. (2000)
	Activity	NC	Klein et al. (1995); Krook et al. (2000)
		↓	Arner et al. (1987); Maegawa et al. (1991); Nolan et al. (1994)
IRS-1	Phosphorylation	↓	Goodyear et al. (1995); Bjornholm et al. (1997); Krook et al. (2000); Pratipanawatr et al. (2001); Aguirre et al. (2002)
PI3K	Phosphorylation	↓	Goodyear et al. (1995); Bjornholm et al. (1997); Kim et al. (1999); Krook et al. (2000)
AKT	Thr phosphrylation	↓	Krook et al. (1998); Karlsson et al. (2005)
AS160	Protein level	NC	Karlsson et al. (2005)
	Phosphorylation	↓	Karlsson et al. (2005)
GLUT4	Protein level	NC	Garvey et al. (1998)
	Translocation	↓	Ryder et al. (2000)

IR: insulin receptor; IRS-1: insulin receptor substrate-1; PI3K: phosphatidylinositol 3-kinase; AKT: V-akt murine thymoma viral oncogene; AS160: AKT substrate of 160 kDa; GLUT4: facilitative glucose transporter 4. ↑: increase; ↓: decrease; NC: no change. Materials compiled from various sources as indicated.

AKT, AKT substrate of 160 kDa (AS160) and glucose transporter 4 (GLUT4)

AS160 is the GTPase-activating protein linking insulin-stimulated PI3K-dependent signalling to GLUT4 translocation (Larance et al., 2005). The expression of GLUT4 protein was shown to be unchanged in type 2 diabetes (Garvey et al., 1998). However, reduction of GLUT4 translocation to the cell surface was reported in skeletal muscle of type 2 diabetic patients, consistent with an impairment in insulin-stimulated AS160 phosphorylation (Ryder et al., 2001, Karlsson et al., 2005). These defects were attributed to a reduction in Thr^{308} phosphorylation of the upstream serine/threonine kinase, AKT (Krook et al., 1998, Karlsson et al., 2005), which may, in turn, be explained by impaired signalling events at upstream levels of IRS-1/PI3K (Karlsson et al., 2005) (summarised in **Table 2**).

1.1.4 Flavonoids

1.1.4.1 Flavonoid biochemistry

Flavonoids are a broad collection of polyphenolic compounds that are found ubiquitously in foods of plant origin (Hollman and Katan, 1999). They contain a C_6-C_3-C_6 carbon framework, consisting of two benzene rings, designated as A and B, connected by a three carbon chain (**Figure 5**), which is closed in most flavonoids to form the heterocyclic ring C (Stafford, 1990, Grotewold, 2006). Depending on the position of linkage of the B-ring to the benzopyran moiety and the degrees of saturation and oxidation of the heterocyclic C-ring, flavonoids are classified into six major subgroups, flavones, flavonols, flavanones, flavanols, anthocyanidins and isoflavones (**Figure 6**). **Figure 7** shows the structures of the representative flavonoids, quercetin, cyanidin and cyanidin-3-*O*-glucoside.

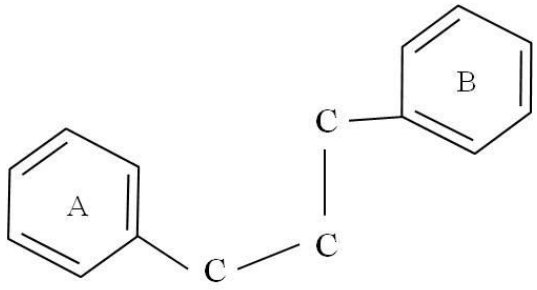

Figure 5. The basic C6-C3-C6 carbon skeleton of flavonoids
The basic C6-C3-C6 unit contains two aromatic rings, A and B, connected by a three-carbon (C) chain. Adapted from Geissman and Hinreiner (1952).

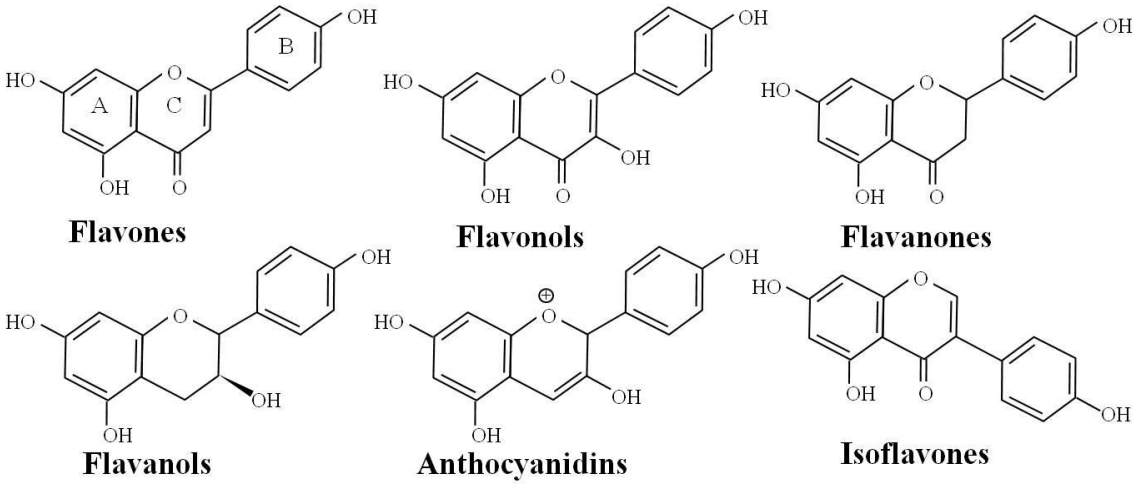

Figure 6. Molecular structures of the six flavonoid subgroups
Flavonoids are divided into six subgroups- flavones, flavonols, flavanones, flavanols, anthocyanidins and isoflavones. Adapted from Hollman and Katan (1999).

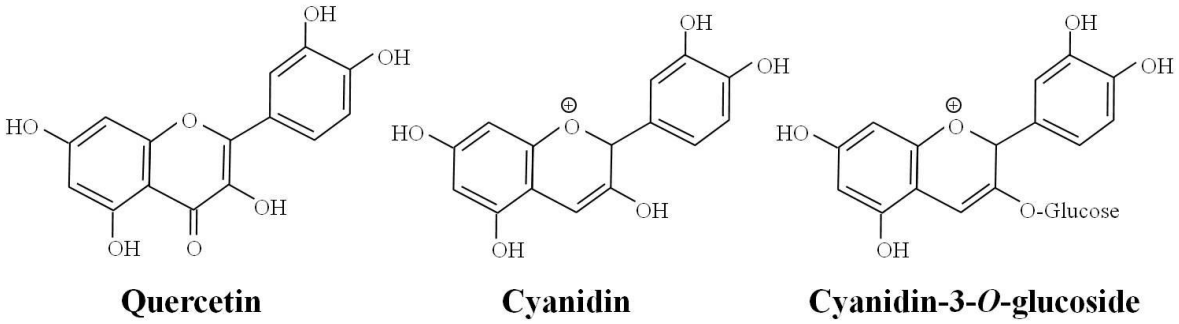

Figure 7. Molecular structures of three representative flavonoids- quercetin, cyanidin and cyanidin-3-*O*-glucoside
Adapted from Hollman et al. (1997) and Tsuda et al. (2003).

Flavonoids are susceptible to a variety of substitutions which include glycosylation, hydrogenation, hydroxylation, malonylation, methylation, prenylation and sulphation (Harborne, 1986, Stafford, 1990). The resulting differences in the chemical structure and relative orientation of various moieties on the molecule determine the biological activities of flavonoids and their metabolites (Aherne and O'brien, 2002). The conjugation patterns also modify the hydrophilicity of the flavonoids. Flavonoid molecules without any sugar moieties are referred to as the aglycone form, whereas those attached to sugar moieties are called flavonoid glycosides, which are more polar.

1.1.4.2 Flavonoid dietary intake

Fruits, vegetables, and beverages such as tea and red wine are especially rich sources of flavonoids (Hertog et al., 1992, Hertog et al., 1993b, Crozier et al., 1997) (**Table 3**). Most of the flavonoids exist in plants with sugars attached as glycosides, although occasionally they are found as aglycones (Prior et al., 2006). Previous studies that reported dietary flavonoid intakes and flavonoid content in food were mostly based on the measurement of only three flavonols, namely quercetin, myricetin, and kaempferol, and two flavones, namely apigenin and luteolin. However, the flavonoid profiles in food consumed by human kind are much more diverse than those typically analysed. Therefore, total flavonoid intake and content in foods can be assumed to be greater than those reported (Aherne and O'brien, 2002). **Table 4** shows a list of studies that estimated the flavonoid intakes in several countries.

Table 3. Occurrence of flavonoids in common foods

Flavonoid subclass	Major food sources
Anthocyanidins	Cherries, grapes
Catechins	Apples, tea
Flavanones	Citrus fruits
Flavones	Parsley, thyme
Flavonols	Onions, kale, broccoli, apples, cherries, berries, tea, red wine
Isoflavones	Soybeans, legumes

Reproduced from Hollman and Katan (1997).

Table 4. Estimated average daily flavonoid intake in different countries

Country	Subject	Flavonoid intake (mg/day)	Major source	Reference
Australia	Adult men and women	454	Apples, onions	Johannot & Somerset (2006)
Fiji	Adult men and women	18	Not specified	Lako et al. (2006)
Finland	Adult men	131	Not specified	Mursu et al. (2008)
Germany	Adult men and women	54	Fruit, fruit product, fruit juice	Linseisen et al. (1997)

Japan	Adult women	64	Green tea	Hertog et al. (1995)
The Netherlands	Adult men	26	Black tea, onions, apples	Hertog et al. (1993a)
UK & Ireland	Not specified	182 & 177, respectively	Grapes, oranges, beer, wine, apples, onions and tea	Beking & Vieira et al. (2011)
USA	Adult men and women	20-22	Onions, tea, apples	Sampson et al. (2002)
USA	Adult men and women	190	Tea	Song & Chun (2008)

Materials compiled from various sources as indicated.

1.1.4.3 Flavonoid absorption and metabolism

Dietary flavonoids differ in the arrangements of hydroxyl, methoxy, and glycosidic side groups, and in the conjugation between the A- and B- rings. The most common glycosidic unit is glucose, but other examples include glucorhamnose, galactose, arabinose, and rhamnose (Cook and Samman, 1996). Flavonoids conjugated to different glycosidic units have different metabolic fates and thus separate locations of uptake. For example, quercetin-3-*O*-glucoside is hydrolysed to its aglycone by lactase phloridzin hydrolase found on the brush border of the mammalian small intestine (Day et al., 1998, Day et al., 2000, Sesink et al., 2003). The aglycone is subsequently absorbed in the small intestine (Spencer et al., 1999, Crespy et al., 2001). In contrast, rutin (quercetin-3-*O*-rutinoside) is not hydrolysed in the small intestine but passes through it to enter the cecum and colon, where it is hydrolysed by enterobacteria and absorbed (Macdonald et al., 1983, Bokkenheuser et al., 1987).

Due to the hydrophobic nature of flavonoid aglycones, they can be transported across the phospholipid bilayer of cellular membrane by passive diffusion (Hollman et al., 1995). On the other hand, some evidence suggested that anthocyanidin glycosides are absorbed intact (Cao et al., 2001, Mcghie et al., 2003, Talavéra et al., 2004). The

increased hydrophilicity of the flavonoid glycosides reduces the possibility of passive transport. Instead, the sodium-glucose co-transporter (SGLT1), sodium-dependent vitamin C transporter (SVCT1) and bilitranslocase have been reported to be involved in the active transport of the glycosides (Aherne and O'brien, 2002, Passamonti et al., 2009). It was estimated that total polyphenol concentrations in the gut lumen can reach the lower milimolar range (Williamson, 2013). With a bioavailability of about 1%, the maximum plasma concentrations after single-dose administration of flavonoids to humans were found to be in the low micromolar range (e.g. to a maximum of 5 μM for quercetin-3-*O*-glucoside) (Hollman, 2004). Flavonoids not absorbed in the small intestine pass to the large intestine where they are hydrolysed by colonic microflora and the products absorbed, or otherwise degraded as described below (Hollman et al., 1995, Walle et al., 2000).

After absorption, flavonoids and their derivatives are thought to undergo subsequent reactions such as hydroxylation, methylation, reduction or conjugation to glucuronide or sulphate either in the intestine or in the liver (Hackett, 1986, Zhang et al., 2007). The flavonoid metabolites that circulate in the blood stream are bound to albumin (Terao et al., 2008). Flavonoid glucuronides and sulfates are readily excreted by mammals in urine and bile as they are polar and water soluble. When excreted in bile, the flavonoid metabolites are passed into the duodenum to be metabolised by intestinal microflora, which either hydrolyse them to the aglycone form to be reabsorbed, or degrade them to monophenolic acids by cleaving the flavonoid ring system (Hackett, 1986, Schneider et al., 1999).

1.1.5 Flavonoids and glucose transport

Flavonoids are ubiquitously found in fruits and vegetables, as well as certain beverages, such as tea and wine. Studies have suggested that flavonoids are beneficial in protecting against many chronic diseases, such as cancers, cardiovascular diseases and neurological disorders (Hertog, 1996, Yao et al., 2004, Letenneur et al., 2007). There is also evidence that flavonoids have beneficial effects on modifying glucose absorption and are thus regarded as potential anti-diabetic agents (Kwon et al., 2007). In fact, a recent cohort study suggested that a higher consumption of anthocyanins and anthocyanin-rich fruits (comparison of ≥ 2 servings/week with < 1 serving/month) was associated with a lower risk of type 2 diabetes in US adults (Wedick et al., 2012).

1.1.5.1 Flavonoids and intestinal glucose transport

Previous scientific studies have investigated the potencies of various flavonoids to alter glucose transport in the intestine as well as in other tissues, for instance, adipocytes and muscle cells (Cao et al., 2007, Lee et al., 2010a, Martel et al., 2010). In the intestine, some of the flavonoids, including anthocyanidins, catechins and flavonols and their conjugated counterparts were shown to inhibit glucose uptake, which will be discussed below.

Effect of pure flavonoids on intestinal glucose transport

As mentioned previously, transport of glucose into intestinal cells is mediated by the sodium-glucose co-transporter SGLT1, and the facilitative glucose transporter GLUT2 located at the apical membrane. Release of glucose from the enterocytes into the blood circulation is accomplished by GLUT2 at the basolateral membrane. Previous

investigations have demonstrated that certain flavonoids disturb intestinal transport of glucose by interacting with either or both of these transporters.

Welsch et al. (1989) showed that the phenolic compounds, tannic acid, catechol, catechin, chlorogenic, ferulic and caffeic acids, were able to reduce glucose uptake into SGLT1-containing brush border membrane vesicles (BBMV) isolated from rat small intestine, but gallic acid had a negligible effect. Two other studies using similar approaches demonstrated that both quercetin-3-*O*-glucoside and quercetin-4'-*O*-glucoside reduced sodium-dependent intestinal glucose uptake, with an increased K_m but only slight changes in V_{max} (Ader et al., 2001, Cermak et al., 2004). In contrast, quercetin-3-*O*-galactoside, quercetin-3-*O*-rutinoside, the aglycone quercetin, as well as naringenin-7-*O*-glucoside, genistein-7-*O*-glucoside and cyanidin-3,5-*O*-diglucoside did not cause any effects (Ader et al., 2001, Cermak et al., 2004). Taken together, it was believed that quercetin glucosides inhibited intestinal glucose uptake by competitive inhibition of SGLT1 and that the positions of glucose residues on the quercetin were unimportant (Ader et al., 2001, Cermak et al., 2004).

To investigate the interaction between flavonoids and the intestinal facilitative glucose transporters, Chen et al. (2007) measured glucose uptake using GLUT2-containing basolateral membrane vesicles (BLMV) isolated from rat jejunum. Most flavonoids tested (both glycosides and aglycones), which included epicatechin gallate (ECg), quercetin-3-*O*-glucoside, fisetin and gossypin, inhibited glucose uptake in BLMV in a competitive manner (Chen et al., 2007). These results indicated that the intestinal facilitated glucose transporter recognised a variety of flavonoids with or without conjugation.

A wider range of polyphenols were tested on their ability to alter intestinal glucose uptake in the Caco-2 cell line (Johnston et al., 2005). Under sodium-dependent conditions which favour uptake via SGLT1, glucose uptake was inhibited by the flavonoid glucosides neohesperidin dihydrochalcone and phloridzin, as well as by the non-glycosylated dietary polyphenols, catechin, epicatechin, epigallocatechin gallate (EGCg), epicatechin gallate (ECg) and epigallocatechin (EGC). However, the aglycones and phenolic acids, caffeic acid, chlorogenic acid, ellagic acid, gallic acid, *p*-coumaric acid and ferulic acid did not alter glucose uptake (Johnston et al., 2005). Under sodium-independent conditions that favours GLUTs-mediated transport, the polyphenol aglycones, phloretin, quercetin, apigenin and myricetin, and also the non-glycosylated dietary polyphenols, EGCg, ECg and EGC caused significant reductions in glucose uptake, while the glucosides and phenolic acids were ineffective (Johnston et al., 2005). These aforementioned authors believed that the aglycones inhibited facilitated glucose uptake whereas glycosides inhibited the active transport of glucose. The non-glycosylated dietary polyphenols appeared to exert their effects via steric hindrance (Johnston et al., 2005).

In addition to using intestinal models with naturally present glucose transporters, Kwon et al. (2007) studied the uptake of 2-deoxyglucose and fructose, both of which are substrates of the GLUT2 transporter, into *Xenopus* oocytes or pituitary cells injected with human GLUT2 cRNA. Both 2-deoxyglucose and fructose were inhibited by quercetin aglycone, quercetin-3-*O*-glucoside and quercetin-4'-*O*-glucoside, but not quercetin-3-*O*-rutinoside (Song et al., 2002, Kwon et al., 2007). However, neither the aglycone nor the glycosides of quercetin had any effects on glucose or fructose transport into *Xenopus* oocytes when treated to express SGLT1 or GLUT5 (intestinal fructose transporter) (Kwon et al., 2007).

Effect of flavonoid-rich food extracts on intestinal glucose transport

In addition to investigating the inhibitory effects of pure flavonoids on intestinal glucose transport, researchers have also tested the efficacy of various food extracts that are rich in flavonoids.

Tea has one of the highest contents of flavonoids among common food and beverage products and catechins comprise a major proportion of its polyphenolic content (Peterson et al., 2005). Makoto et al. (2000) observed an inhibitory effect of tea extracts (green, black, roasted and oolong teas) on glucose uptake in Caco-2 cells. Further investigations of glucose uptake using pure tea polyphenols showed that catechins possessing the galloylester group, i.e. ECg, EGCg and theaflavins, inhibited glucose uptake but those without, i.e. epicatechin, EGC and thearubidin, did not. In BBMVs from rabbit small intestine, which contain SGLT1 as the major glucose transporter, ECg was not transported by SGLT1 but inhibited glucose transport via SGLT1 in a competitive manner (Kobayashi et al., 2000, Makoto et al., 2000). Wang et al. (2008) also reported inhibition of glucose uptake, regardless of Na^+ dependency, by aqueous extracts of Kuding tea, chrysanthemum and purple sweet potato stem, in Caco-2 cells. The authors suggested that the constituent dicaffeoylquinic acids and flavanols contributed to the inhibitory effects of the abovementioned beverages (Wang et al., 2008).

Fruits are good sources of flavonoids such as anthocyanins and flavonols. Chronic exposure (96 hours) of anthocyanins extracted from red grape skin was found to increase the mRNA expression of GLUT2 in Caco-2 cells (Faria et al., 2009). However, glucose uptake decreased in the presence of anthocyanins. This was possibly

due to competition between glucose and anthocyanins with glucose residues for the GLUT2 transporter. This was confirmed by the ability of malvidin-3-glucose but not the aglycone malvidin to inhibit glucose uptake (Faria et al., 2009). Moreover, extracts of strawberry and apple also inhibited uptake of glucose into Caco-2 cells, as well as transport of glucose from the apical to the basolateral side of Caco-2 monolayers (Manzano and Williamson, 2010). The inhibition of GLUT2 was greater than SGLT1, a supposition deduced from studies which compared transports under sodium-containing and sodium-free conditions. The constituent polyphenols, quercetin-3-*O*-rhamnoside, phloridzin and 5-caffeoylquinic acid, were shown to contribute to the glucose transport inhibitory activity of the apple extract. In contrast, pelargonidin-3-*O*-glucoside contributed to the inhibitory effect of the strawberry extract (Manzano and Williamson, 2010).

One study has systematically screened medicinal plants that have putative anti-diabetic and anti-obesity activities to naturally treat type 2 diabetes and obesity in Canadian native populations (Nistor Baldea et al., 2010). Thus, crude ethanol extracts of seventeen Boreal forest medicinal plants, which were rich in flavonoids such as quercetin, were tested for their inhibitory effect on intestinal glucose absorption *in vitro* using Caco-2 cells (Nistor Baldea et al., 2010). Extracts from thirteen of these medicinal plants tested acutely inhibited the uptake of deoxyglucose in Caco-2 cells during co-incubation, whilst five extracts exerted an inhibitory effect after a 6-hour pre-incubation. Western blot analysis demonstrated that the reduction in glucose uptake might be explained by a decrease in the protein expression of GLUT2 or SGLT1 glucose transporters (Nistor Baldea et al., 2010).

Animal studies

Skopec et al. (2010) evaluated the effect of various flavonoids (i.e. quercetin, quercetin-3-*O*-glucoside and phloridzin in rats and American robins, and naringenin, naringenin-7-glucoside, genistein, epigallocatechin gallate (EGCg), and phloretin in rats only) on the bioavailability of the non-metabolisable analogue of glucose, 3-*O*-methyl D-glucose (3MG). None of the flavonoids tested in robins significantly decreased the bioavailability of 3MG. This can be explained by the fact that small birds such as robins rely largely on non-mediated intestinal absorption of glucose through the paracellular pathway. On the other hand, mammals such as rats absorb glucose across the enterocytes using glucose transporters, SGLT1 and GLUT2. Six of eight flavonoids (i.e. quercetin-3-*O*-glucoside, phloridzin, naringenin, naringenin-7-glucoside, EGCg and phloretin) inhibited 3MG uptake in rats (Skopec et al., 2010).

Human studies

In addition to using *in vitro* models and animals for investigating the effect of flavonoids on intestinal glucose transport, human trials have also been carried out to study the glycaemic effect of flavonoid-rich foods.

Consumption of commercially available apple juices, clear or cloudy, was demonstrated to trigger delays in glucose absorption as shown by a reduction in the incremental area under the curve following juice consumption, compared to consumption of a control beverage with an adjusted sugar content (Johnston et al., 2002). Consistent with the delay in intestinal glucose absorption, proximal intestinal secretion of the gut hormone glucose-dependent insulinotropic polypeptide (GIP) was suppressed whilst secretion of glucagon-like peptide-1 (GLP-1) in the distal region of the gut was enhanced (Johnston

et al., 2002). The aforementioned authors attributed the delaying effect on intestinal glucose absorption by apple juice to the well-established SGLT1 inhibitor, phloridzin, without excluding the possibility of modification by phenolic acids present in the beverage. A similar study on coffee carried out by the same research group also showed delayed intestinal glucose absorption, which was observed after consumption of either caffeinated or decaffeinated coffee compared to control beverage (Johnston et al., 2003). This effect was believed to be exerted by the coffee polyphenol, 5-caffeoylquinic acid. In contrast, the increased postprandial plasma glucose after consumption of caffeinated coffee compared to decaffeinated coffee or control beverage might suggest a mildly impaired glucose tolerance caused by caffeine (Johnston et al., 2003).

Törrönen et al. (2010) examined the glycemic effect of a mixed berry puree made of bilberries, blackcurrants, cranberries and strawberries, sweetened with sucrose in healthy human subjects. In subjects who consumed the berry meal, the time to reach the peak plasma glucose concentration was delayed by 15 minutes after consumption of the berry meal, whilst the peak plasma glucose increase from baseline was also reduced, compared to the control meal. It was suggested that berries rich in polyphenols had an effect of delaying and reducing the postprandial glucose response of sucrose (Törrönen et al., 2010). The delayed postprandial glucose response due to the mixed berries puree was similar to the results of another study carried out by the same group using blackcurrant and lingonberry puree or nectar (Törrönen et al., 2012a). They further demonstrated that the berry meals stimulated a lower and prolonged insulin response compared with control meals, whilst plasma free fatty acid concentrations were suppressed (Törrönen et al., 2012a).

Another study further investigated the effect of consumption of berries on the postprandial glycaemic response to a solid starchy food (Clegg et al., 2011). The results showed that consumption of pancakes supplemented with either raspberries or blueberries did not alter either the glycaemic response, glycaemic response area under the curve nor the satiety index in healthy human subjects, as compared to consumption of control pancakes containing similar amounts of fructose and glucose. This indicated that the ability of berries to attenuate blood glucose level in response to starch-based foods, as represented by pancakes in this study, is unsubstantiated (Clegg et al., 2011).

1.1.5.2 Flavonoids and glucose utilisation in diabetic animal models

The efficacy of various flavonoids to modulate plasma glucose level has been studied using a range of animal models. In streptozotocin-induced diabetic rats that lack endogenous insulin, myricetin, a flavonol with a similar structure as quercetin and commonly found in tea, fruits and vegetables, was demonstrated to attenuate plasma glucose levels (Liu et al., 2005). Increases in glucose utilisation in the soleus (type I, aerobic) muscle as well as enhancement of hepatic glycogen synthesis were observed along with decreased plasma glucose levels (Liu et al., 2005). Similarly, an extract from seeds of the tropical fruit jambul, which is rich in flavonoids such as rutin and quercetin, also improved glucose tolerance, glycogen biosynthesis and glucose uptake in streptozotocin-induced diabetic mice (Sharma et al., 2008).

Feeding experimental animals with a high fructose diet was demonstrated to induce glucose intolerance and hyperinsulinaemia, which mimics type 2 diabetic conditions (Huang et al., 2004). Intravenous injection of myricetin improved insulin sensitivity in high-fructose chow-fed rats as demonstrated by a reduction in high plasma glucose and

triglyceride levels induced by the high-fructose diet (Liu et al., 2007). On the other hand, in young genetically insulin-resistant obese KKA^{y} mice, administration of a bio-transformed blueberry juice reduced food intake and body weight, protecting them against the subsequent development of glucose intolerance and the onset of diabetes (Vuong et al., 2009).

1.1.6 MicroRNA: from biogenesis to degradation

MicroRNAs (miRNAs) are a family of short single-stranded non-coding RNAs, of about 22 nucleotides in length. They extensively regulate gene expression post-transcriptionally in animals, plants and protozoa. It is believed that in mammals, miRNAs can control the expression of more than 60% of all protein-coding genes (Fabian et al., 2010). Specifically, miRNAs have been implicated in the control of glucose homeostasis and the pathogenesis of diabetes (Tang et al., 2008, Frost and Olson, 2011). Therefore, it would seem reasonable that the repeated changes in mRNA expression due to the exposure of cells to berry extract may be initiated firstly by changes in miRNA rather than direct actions on the synthesis of mRNA via transcription. In other words, the changes in miRNA allow for a more in-depth analytical insight into hypothesis-driven research that examines nutrient-nucleic acid interactions.

1.1.6.1 MicroRNA biogenesis

Genomic distribution

There are several types of genomic distribution of miRNA genes. Some exist as clusters throughout the genome, which are transcribed as polycistronic primary transcripts, resulting in multiple miRNAs (Lee et al., 2002). Some are transcribed as

independent units from genomic positions that lie within the introns or extrons of other protein-coding or non-coding transcripts (Rodriguez et al., 2004), while the others are transcribed from intergenic regions (Lee and Ambros, 2001, Grad et al., 2003).

MicroRNA transcription

MicroRNAs are generally believed to be transcribed by RNA polymerase II as primary miRNA transcripts (pri-miRNAs) that contain a 5'-end 7-methylguanosine cap structure and a 3'-end poly(A) tail, which are unique characteristics of class II gene transcripts (Lee et al., 2004) (see **Figure 8**). However, Borchert and colleagues revealed that miRNAs in the human chromosome 19 miRNA cluster are interspersed among Alu repeats. Since Alu transcription occurs by recruiting RNA polymerase III (pol III), they suggested that these miRNAs are transcribed by pol III (Borchert et al., 2006).

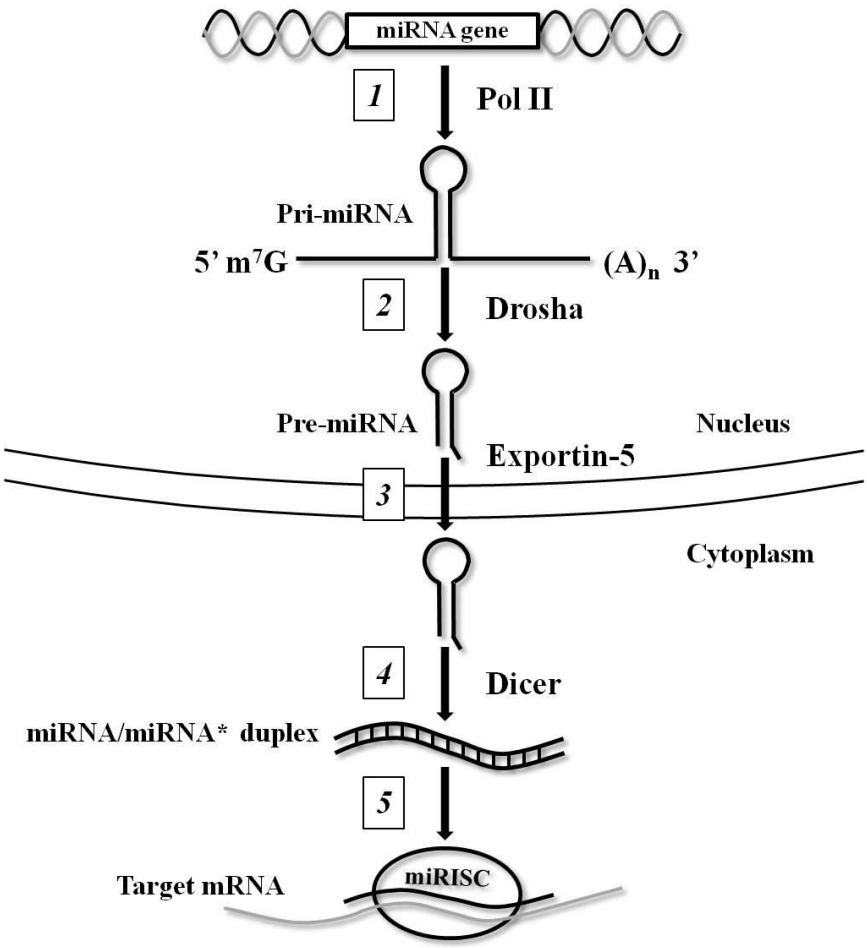

Figure 8. Biogenesis of miRNA

1) miRNA is transcribed as a primary miRNA transcript (pri-miRNA) that contains a 5'-end 7-methylguanosine cap structure (5'm^7G) and a 3'-end poly(A) tail ($(A)_n$3'), presumably by RNA polymerase II (Pol II); ***2)*** pri-miRNA is cleaved into a ~70-nucleotide long stem loop structure called precursor miRNA (pre-miRNA) by the endonuclease, Drosha; ***3)*** pre-miRNA is exported to the cytoplasm by exportin-5; ***4)*** cytoplasmic cleavage by another endonuclease, Dicer, gives rise to a miRNA/miRNA* duplex; ***5)*** the less stable strand of miRNA from the duplex form the miRNA-induced silencing complex (miRISC) with effector proteins including Argonaute proteins. Binding of miRISC to target mRNA leads to its post-transcriptional regulation. Adapted from Bartel (2004) and Murchison and Hannon (2004).

Processing of miRNA transcripts

MicroRNAs are transcribed as several kilobase-long pri-miRNAs bearing hairpin-shaped structures. These pri-miRNAs are cleaved by Drosha, an RNase III endonuclease enzyme that complexes with the double-stranded RNA binding domain protein DGCR8 (Lee et al., 2003). The transcripts are released as an approximately 70-

nucleotide long stem loop structures called precursor miRNAs (pre-miRNAs), with a 2-nucleotide long 3'-end flanking region (Zeng and Cullen, 2004). Pre-miRNAs are subsequently exported, by exportin-5, out of the nucleus to the cytoplasm (Kim, 2004, Lund et al., 2004). Further cytoplasmic processing by another RNase III enzyme, Dicer, assisted by the transactivation-responsive RNA binding protein (TRBP), cleaves the pre-miRNAs to generate miRNA/miRNA* duplexes about 22-nucleotide in length (Hutvagner et al., 2001). Depending on the thermodynamic stability of the 5' end, the less stable strand will be selected as the guide strand to be incorporated into the miRNA-induced silencing complex (miRISC), which usually contains Argonaute (AGO) proteins and glycine-tryptophan protein of 182 kDa (GW182) as the effector components (Schwarz et al., 2003). The passenger strand that remains from the miRNA/miRNA* duplex, on the other hand, is released and degraded (Schwarz et al., 2003).

1.1.6.2 Mechanisms of miRNA-mediated regulation

Target recognition

After a series of cleavage events and incorporation into the miRISC, the miRNAs pair to sites in mRNAs to direct post-transcriptional regulation. In plants, miRNAs might hybridise to any regions on their target mRNAs with perfect or near-perfect complementarity and often trigger mRNA cleavage (Rhoades et al., 2002). On the other hand, in animals, functional miRNA-target pairing can be more variable with mismatches and bulges, and contain only short stretches of complementary sequence (Brennecke et al., 2005). Most of these matching involve interaction between the seed regions (nucleotides 2-7 from 5' end) of miRNAs and the conserved sites in the 3' untranslated regions (3'UTR) of mRNAs (Lewis et al., 2003). Less commonly,

miRNAs may also target 5'UTR or coding regions of mRNAs (Orom et al., 2008, Gu et al., 2009).

In addition to the complementarity of the seed region, an A residue across from miRNA nucleotide 1 and an A or U residue across from nucleotide 9 were found to improve miRNA target recognition (Lewis et al., 2005, Nilsen, 2007). Pairing to the 3' region of the miRNA helps stabilise the interaction between the miRNA and the mRNA. Positioning of the functional site within a high local AU content also increases site efficacy (Grimson et al., 2007, Nilsen, 2007).

Modes of regulation

MicroRNAs mediate regulation of target mRNAs via multiple mechanisms, which include 1) cleavage of mRNA transcripts (slicing, which requires perfect or near-perfect complementarity); 2) repression of mRNA translation; 3) destabilisation through de-adenylation and 4) localisation in the processing bodies (P-bodies) (Nilsen, 2007, Jung et al., 2009).

mRNA cleavage (slicing)

For miRNAs that bind to their target mRNAs with perfect or near-perfect complementarity, as in the case of most plant miRNAs, mRNA cleavage is believed to be a predominant mechanism by which gene expression is regulated (Carrington and Ambros, 2003). To trigger mRNA cleavage, the AGO component of miRISC cleave a single phosphodiester bond of the target mRNA that pairs to the nucleotides 10 and 11 of the miRNA (Hutvágner and Zamore, 2002, Llave et al., 2002). The cleaved

fragments are then released and degraded, freeing the miRISC to recognize and regulate another target mRNA (Jung et al., 2009).

Translational repression

Direct effects of miRNAs on translation may occur at different stages of this process. It might be caused by the impairment of cap recognition in the initiation of protein synthesis, causing a defect in ribosome recruitment to the mRNA at the initiation stage (Pillai et al., 2005). It might also occur at the post-initiation stage, in which the elongating peptide is degraded concomitantly with translation, or premature ribosomes drop off during elongation, leading to premature termination of translation (Humphreys et al., 2005, Nottrott et al., 2006, Petersen et al., 2006).

De-adenylation of mRNA

GW182 protein of the miRISC brings about mRNA de-adenylation of the 3' poly(A) tail and degradation of mRNAs by recruiting deadenylase complex to the target mRNA (Behm-Ansmant et al., 2006). Following de-adenylation, the 5'-end 7-methylguanosine cap structure of the mRNA is removed by de-capping complex and the 5'→3' exonuclease directs exonucleolytic degradation of the mRNA (Garneau et al., 2007, Chekulaeva and Filipowicz, 2009).

mRNA sequestration in P-bodies

The deadenylation-decapping degradation process of mRNAs mediated by miRNAs is believed to localise in cytoplasmic foci called P-bodies (Kulkarni et al., 2010). In addition to serving as a site for mRNA decay, there is also evidence that P-bodies can

act as a repository for repressed mRNAs, where they are sequestered from ribosomes for translation (Brengues et al., 2005, Bhattacharyya et al., 2006). The stored mRNAs can be released from the P-bodies and can undergo translation again (Brengues et al., 2005, Bhattacharyya et al., 2006).

1.1.6.3 MicroRNA degradation

MicroRNA regulation of mismatch target mRNAs is multiple-turnover, i.e. each miRNA incorporated into the miRISC can direct multiple rounds of target mRNA regulation (Hutvágner and Zamore, 2002). Target regulation can increase the miRNA's rate of decay in a manner that is dependent on target concentration and complementarity. It was suggested that target interaction could lead to uridylation of the miRNA , accelerating its decay (Baccarini et al., 2011).

The 5'→3' exonuclease XRN-2 catalyzes the degradation of single-stranded mature miRNAs that fail to be incorporated into AGO proteins or that are released from the effector complex. Interaction of the miRNA-AGO complex with its target prevents release and subsequent destabilisation of the miRNA (Kai and Pasquinelli, 2010).

1.1.7 The use of Caco-2 cells as a model for intestinal epithelial glucose transport

Caco-2 is a cell line isolated from human colorectal adenocarcinoma, which was established in the 1970s originally characterised for cancer studies (Fogh et al., 1977). In culture, this cell line differentiates spontaneously at confluence to form a monolayer of highly polarized cells, which develop microvilli expressing small intestine hydrolase enzyme activities (i.e. sucrose-isomaltase, lactase, aminopeptidase N,

dipeptidylpeptidase IV) on the apical membrane, and tight junctions between adjacent cells. These resemble morphological and functional characteristics of small intestine enterocytes (Pinto et al., 1983). However, because of their colonocytic tumour origin, differentiated Caco-2 cells also exhibit some of the characteristics of colon crypt cells, including electrical properties and ionic conductivity (Grasset et al., 1984).

1.1.7.1 Choices of intestinal epithelial models

To investigate the characteristics of absorption of nutrients and orally administered therapeutic agents at the intestinal level *in vitro*, several cultured intestinal epithelial cell models have been used. These include primary cell cultures, cell lines from normal tissue and established cell lines of tumour origin (reviewed in Sambruy et al., 2001).

Among these cell models, the Caco-2 cell line has been suggested to be one of the best models for intestinal absorption studies (Sambruy et al., 2001). This is because primary intestinal cell cultures obtained either from the small intestine or colon lose their differentiated characteristics rapidly once they are isolated (Fonti et al., 1994). It is also difficult to obtain reproducible and quantitative data from the use of primary cultures (Sambruy et al., 2001). The IEC cell lines were derived from normal small intestine of newborn and germ-free rats (Quaroni et al., 1979). They express some typical epithelial markers but do not form functional tight junctions between adjacent cells, nor develop a fully polarized columnar morphology. They also do not express significant levels of the small intestinal disaccharidases and peptidases and thus are regarded as a cell line that resembles proliferative crypt cells (Quaroni et al., 1979). Comparing the morphological and biochemical characteristics of different cell models, Caco-2 is suggested to be the best model for studies of intestinal absorption (Quaroni and Hochman, 1996).

1.1.7.2 Caco-2 subclones

The Caco-2 cell line is characterised by a heterogeneous population with respect to the differences in morphological and functional differentiation (reviewed in Sambuy et al., 2005). In order to improve homogeneity of the cell population to suit different studies, several clonal cell lines were isolated and characterized. Among different subclones, one of the low glucose-consuming clones, Caco-2/TC7, was found to express concomitantly the sodium/glucose co-transporter SGLT1, the facilitative glucose transporter GLUT2 and the facilitative fructose transporter GLUT5 (Mahraoui et al., 1994a), which are normally present in human small intestinal enterocytes, making it more suitable for intestinal glucose transport studies.

1.1.7.3 Distribution of glucose transporters in Caco-2 cells

In the small intestine, glucose is transferred across the enterocyte apical membrane by the facilitated glucose transporter GLUT2, in addition to the sodium-dependent SGLT1 active glucose transporter (Kellett and Helliwell, 2000), which was once considered to be the only transporter that carried glucose from the intestinal lumen into the enterocytes (Shirazi-Beechey, 1995). Grefner et al. (2010) evaluated and compared the distributions of SGLT1 and GLUT2 in enterocytes of rat small intestine and Caco-2 cells by an immunocytochemical analysis using confocal microscopy. In rat enterocytes, localization of SGLT1 was revealed to be at the edge of intestinal villi, while GLUT2 was observed in both the apical and basal parts of the cells (Grefner et al., 2010). On the other hand, in Caco-2 cells, SGLT1 was found to distribute throughout the entire cytoplasm thickness but more in the apical and subapical parts of the cells, while GLUT2 was located predominantly in the basal area and was absent in the apical area, which is at variance with the distribution in rat enterocytes (Grefner et al., 2010). Nevertheless, Tobin et al. (2008) was able to observe co-localisation of GLUT2 and

sucrase-isomaltase on the apical membrane in Caco-2/TC7 subclone. These studies confirmed that the Caco-2/TC7 subclone is suitable as an *in vitro* cell model for intestinal epithelial glucose transport studies.

1.1.7.4 GLUT2 translocation

In response to high luminal concentrations of glucose (≥ 25 mM), it is believed that a mechanism involving the translocation of cytosolic GLUT2 into the apical membrane of enterocytes occur within a few minutes, allowing the enterocyte to rapidly increase its glucose absorption capacity to match dietary intake (Mace et al., 2007a, Morgan et al., 2007).

Zheng et al. (2012) explored the mechanisms of glucose uptake in several intestinal epithelial cell lines, including Caco-2 cells. They demonstrated that in Caco-2 cells, at low glucose concentrations (≤ 10mM), glucose molecules entered the cells through SGLT1 and GLUT2 transporters that were constitutively located on the apical membrane. When the cells were exposed to high glucose concentrations (≥ 25mM), both K_m and V_{max} of glucose uptake increased within ten minutes. This rapid increase in carrier-mediated glucose absorption required the activation of PKC and an intact cytoskeleton, supporting the theory of intracellular trafficking of GLUT2 (Zheng et al., 2012). This process in Caco-2 cells is consistent with the *in vivo* model of glucose transport.

1.1.8 The use of C2C12 cells as a model for glucose transport in skeletal muscle cells

1.1.8.1 Origin of the C2C12 cell line

The mouse myogenic cell line C2 is an immortal line of mouse skeletal myoblasts originally obtained by Yaffe and Saxel (1977) from satellite cells harvested from the thigh muscle of a two-month-old female C3H mouse 70 hours after a crush injury. The diploid subclone, C2C12, was isolated and karyotyped by Blau et al. (1985) for its ability to differentiate rapidly and produce extensive contracting myotubes expressing characteristic muscle proteins. The cells are adherent in culture and undergo differentiation to form multi-nucleated myotubes when the growth factor content in the cell culture medium is reduced, which can be achieved by switching supplemental fetal bovine serum to horse serum.

1.1.8.2 The insulin signalling pathway in C2C12 cells

Differentiated C2C12 myotubes display carrier-mediated glucose uptake and this is further stimulated by insulin (Sarabia et al., 1990). This aforementioned study provides evidence that C2C12 myotubes possess intact cellular machinery for insulin signalling and additional studies which followed have shown that this occurs via the IRS/PI3K/AKT pathway. Indeed, exposure of C2C12 cells to insulin induces phosphorylation and activation of IR (Wang et al., 2007), IRS-1 (Berti et al., 1997, Wang et al., 2007), PI3K (Berti et al., 1997) and AKT (Schmitz-Peiffer et al., 1999, Wang et al., 2007), and the subsequent stimulation of glucose uptake (Berti et al., 1997, Doi et al., 2003) as well as glycogen synthesis (Schmitz-Peiffer et al., 1999).

In addition to the activation of the PI3K signalling cascade by insulin, intriguingly, chronic exposure of C2C12 to insulin also results in reduced tyrosine phosphorylation

of IRS-1 and subsequent impairment of PI3K activation and insulin-stimulated glucose uptake (Kumar and Dey, 2003). This development of insulin resistance in C2C12 by insulin itself is suggested to be a consequence of serine/threonine phosphorylation of IRS-1 by insulin, leading to reduced tyrosine phosphorylation and degradation of IRS-1 proteins (Pederson et al., 2001, Mussig et al., 2005).

The insulin signalling pathway has also been shown to be affected by a number of analytes. These include the protein hormones, IGF-1 (Rommel et al., 2001), leptin (Berti et al., 1997) and adiponectin (Wang et al., 2007), the branched-chain amino acid isoleucine (Doi et al., 2003), long-chain saturated fatty acids such as palmitate (Schmitz-Peiffer et al., 1999, Chavez and Summers, 2003) and cytokine TNF-α (Rui et al., 2001).

1.1.8.3 The AMPK pathway in C2C12 cells

Contraction-stimulated glucose uptake in skeletal muscle is regulated by an insulin-independent signalling pathway (Hayashi et al., 1998). This distinct signalling mechanism, which is activated by an increase in the AMP/ATP or ADP/ATP ratios as well as intracellular Ca^{2+} concentrations, is identified as the AMPK pathway (Wright et al., 2004). In C2C12 cells, it has been shown that muscle contraction leads to phosphorylation and activation of endogenous AMPK (Nedachi et al., 2008, Niu et al., 2010). The resultant downstream signalling of AMPK induces changes in cellular energy metabolism via stimulation of glucose uptake and fatty acid oxidation (Yamauchi et al., 2002).

1.1.8.4 GLUT4 translocation

Insulin stimulates glucose uptake in C2C12 myotubes and this is accompanied by an increase in the GLUT4 content within the plasma membrane (Galante et al., 1995, Lee et al., 2011). Insulin-induced surface expression of GLUT4 was completely inhibited by the PI3K inhibitor wortmannin (Nedachi and Kanzaki, 2006) strongly suggesting that differentiated C2C12 cells to be endowed with the basic GLUT4 translocation machinery needed to respond to insulin stimulation. Furthermore, contraction- or metformin-induced phosphorylation of AMPK also triggered an increase in cell surface GLUT4 density (Niu et al., 2010, Lee et al., 2011).

1.1.9 Alcohol

Heavy alcohol consumption is associated with increased morbidity and mortality (Preedy and Watson, 2005, Watson et al., 2013). Although the lifestyles typical to heavy drinkers might contribute to the abovementioned association (for instance, poor nutrition and injuries attributed to excessive drinking) (Morgan, 1982, Cherpitel, 1993), the toxicological effects of alcohol (and/or its metabolite, acetaldehyde) *per se* account for a substantial part of the increased risk of medical conditions (Darke et al., 2013, Watson et al., 2013). Previous studies have shown that chronic alcoholism is associated with higher risks of various human diseases, to name a few, alcoholic cardiomyopathy, alcoholic liver diseases, alcoholic myopathy, peripheral neuropathy and cancers (Martin et al., 1985, Komajda et al., 1986, Sheehy, 1992, Monforte et al., 1995, Walsh and Alexander, 2000, Bagnardi et al., 2001).

1.1.9.1 Alcoholic myopathy

Alcoholic skeletal myopathy is one of the most common alcohol-related diseases (see **Table 5**) (Estruch et al., 1993) and occurs in 40-60% of chronic alcoholics (Martin et al., 1985, Urbano-Marquez et al., 1989, Urbano-Marquez et al., 1995). These

susceptible subjects were characterised by gradual loss of muscle mass, muscle weakness and abnormal gait. With respect to molecular mechanism, the reduction of muscle mass is attributed to reduced protein synthesis and subsequent atrophy of muscle fibres (Preedy and Peters, 1988b). This occurs selectively in the type II skeletal muscle fibres (anaerobic, glycolytic, fast-twitch), particularly, in the type II B subset fibres, which have the lowest oxidative capacity and the fewest mitochondria (Hanid et al., 1981, Fernández-Solá et al., 1995, Reilly et al., 2000). The oxidative type I (aerobic, slow-twitch) muscle fibres are protected in early stages but are affected in some individuals after reduction of type II fibre diameters (Slavin et al., 1983).

Table 5. Number of chronic alcoholic patients who had alcohol-related complications

Alcohol-related disesases	Number of cases/ total (%)
No complications	99/250 (44)
Cardiomyopathy	20/232 (9)
Cirrhosis	20/232 (9)
Peripheral neuropathy	41/250 (16)
Skeletal myopathy	117/250 (48)

Patients were chronically alcoholic men entering an alcoholism treatment program in the mentioned clinical study. Adapted from Estruch et al. (1993).

Regulation of transcription and protein synthesis

The c-MYC proto-oncogene encodes for a transcription factor that is involved in the regulation of cell development and growth (Evan et al., 1994). Constitutive expression of c-MYC up-regulates many genes involved in cell proliferation and is implicated in many cancers (Spencer and Groudine, 1991, Evan et al., 1994). Expression of c-MYC has also been shown to increase in muscle hypertrophy (Whitelaw and Hesketh, 1992). Intriguingly, although impairment in protein synthesis has been observed consistently in muscles exposed to ethanol *in vitro* and *in vivo* (Preedy and Peters, 1988a, Hong-Brown et al., 2006), both mRNA and protein levels of c-MYC were up-regulated in rat skeletal

and cardiac muscles in response to alcohol administration (Paice et al., 2002). The authors suggested the increase in c-MYC expression might be a cellular adaptation against the insults of ethanol (Paice et al., 2002).

Since earlier investigations had suggested impairment in translational efficiency as a possible explanation for the alcohol-induced reduction in protein synthesis (Preedy et al., 1988), Lang and co-workers examined the changes of several eukaryotic translational initiation factors (eIFs) in response to alcohol administration in rats (Lang et al., 1999, Lang et al., 2000). In both acute (2.5 hours after intraperitoneal ethanol injection) and chronic studies (14-week ethanol feeding), ethanol induced an increase in the binding of eIF4E protein to the translational repressor 4E-binding protein 1 (4E-BP1) but a decrease in the level of the active eIF4E-eIF4G complex (Lang et al., 1999, Lang et al., 2000). In rats chronically fed with alcohol, a reduction in eIF2B activity was also observed in type II muscles (Lang et al., 1999). Altogether, these suggested the involvement of eIFs in alcohol-induced impairment of muscle protein synthesis.

Myosin and other myofibrillary proteins

Myosin is a motor protein complex composed of two heavy chains and two pairs of light chains. The expression profile of myosin heavy chain isoform proteins exerts a strong influence on the contractile properties of the muscle fibre (Harridge et al., 1996). Interactions between myosin and associated contractile proteins, including actin, troponin and tropomyosin, also play a pivotal role in muscle contraction (El-Saleh et al., 1986).

In chronically ethanol-fed rats, a reduction in the total myofibrillary protein content in the type II fibre-predominant plantaris muscle has been reported (Reilly et al., 2000). A

concomitant reduction in total myosin content, as a consequence of specific declines in the myosin heavy chain (MYH) Ib, IIx and IIb protein isoforms, was observed (Reilly et al., 2000). There were no significant changes in both the total and specific MYH isoform contents in the type I-predominant soleus muscle (Reilly et al., 2000). At the mRNA level, only the expression of MYH-Ib showed significant reduction in response to chronic ethanol feeding, in both muscle types (Reilly et al., 2000).

In the abovementioned study, the researchers also examined the changes in the levels of several other myofibrillary proteins, namely actin, α-actinin, desmin, troponins and tropomyosin, in ethanol-fed rats (Reilly et al., 2000). In soleus muscle, only troponin-C showed a significant decrease, whilst in plantaris muscle, ethanol feeding triggered significant reductions in actin, desmin and troponin-I (Reilly et al., 2000).

Alcoholism and glucose utilisation in muscles

In healthy man, acute infusion of ethanol has been shown to decrease glucose disposal under basal condition as well as during euglycaemic hyperinsulinaemia, indicating development of insulin resistance (Yki-Jarvinen and Nikkila, 1985, Shelmet et al., 1988). Acute ethanol administration to rats demonstrated that this inhibitory effect was tissue-specific, as ethanol infusion attenuated glucose utilisation in skeletal muscles and the heart, but not in diaphragm, lung, liver, skin, ileum and adipose tissue (Spolarics et al., 1994). Xu et al. (1996) demonstrated that the inhibitory effect of acute ethanol exposure on whole-body glucose disposal was associated with marked decreases in glucose uptake and glycogen synthesis in most skeletal muscles. These changes may be explained by a defect in glycogen synthase activation in response to insulin in oxidative muscles by ethanol (Xu et al., 1996).

Beside the acute inhibitory effect of ethanol on glucose utilisation, chronic ethanol exposure has also been shown to impair glucose disposal and/or insulin sensitivity (Onishi et al., 2003, Wan et al., 2005, Qu et al., 2011). Previous studies have suggested an interaction between the insulin signalling and G-protein-mediated pathways (Moxham and Malbon, 1996). Wan et al. (2005) demonstrated that an increase in $G_s\alpha$ (G stimulatory α subunit) expression in muscle of ethanol-fed rats may contribute to the ethanol-induced impairment of insulin signal transduction. Other studies reported inhibition of insulin/IGF/IRS and changes in the downstream PI3K/AKT signalling in muscle in response to ethanol, including reductions in IRS-1 and AKT phosphorylation (Qu et al., 2011, Nguyen et al., 2012). Ethanol-induced defects in the PI3K/AKT signalling cascade have also been reported in other tissues, such as liver and adipose tissue (Yi and Jhun, 2004, He et al., 2006).

In addition to defects in the insulin signalling pathway, alcoholic skeletal myopathy in chronic alcoholic patients is also accompanied by reduced activities of glycogen phosphorylase, phosphofructokinase and fructose 1,6-bisphosphatase, key regulatory enzymes in the anaerobic glycolytic pathway, as well as the subsequent reduction in glycogen content in the muscle biopsies of the patients (Martin et al., 1984).

However, despite the prevalence of alcoholic myopathy, the precise molecular mechanisms remain poorly understood. This may be due to the fact that multiple pathophysiological processes occur in muscle such as adduct formation, altered proteolysis, increased RNase activity, to name but a few (Fernandez-Solà et al., 2007).

1.2 Hypotheses

Previous studies carried out by our research group showed, using mRNA expression microarrays, that flavonoid-rich berry extract exerted cellular effects on multiple membrane transport systems in the intestinal epithelial Caco-2 cell model, including those related to glucose transport. In the present project for this thesis, we aimed to address the hypotheses that,

1. berry extract will acutely affect glucose transport in Caco-2 cells, by exerting direct inhibitory effects of glucose transporter activity;
2. berry extract will chronically affect glucose transport in Caco-2 cells, by suppressing the expression of glucose transporters;
3. the post-transcriptional regulators, microRNAs, are involved in the modulation of glucose transporter expression in Caco-2 cells by berry extract;
4. berry extract can modulate glucose transport in peripheral tissues, as represented by skeletal muscle, using the C2C12 muscle cell model;
5. in addition to glucose transport, berry extract can modulate other cellular functions in C2C12 muscle cells;
6. ethanol alters multiple cellular functions in C2C12 muscle cells;
7. berry extract can protect C2C12 muscle cells from ethanol-induced cell damage.

1.2.1 Objectives

1. To evaluate the acute effects of berry extract and related polyphenols on glucose transport in the intestinal Caco-2 cells by measuring glucose uptake following short-term exposure (15 minutes). This will be performed using [^{3}H]-labelled D-glucose under sodium-containing or sodium-free conditions (jointly with Dr Fawaz Alzaid, another PhD student from our group);

2. To evaluate the chronic effects of berry extract and related polyphenols on glucose transport in Caco-2 cells by measuring changes in the gene expression of glucose transporters and glucose uptake after longer-term exposure (16 hours). Relative gene expression will be measured by qRT-PCR (jointly with Dr Fawaz Alzaid, another PhD student from our group);
3. To investigate the changes in global microRNA expression in Caco-2 cells in response to 16-hour-incubation with berry extract, using Affymetrix GeneChip® miRNA arrays;
4. To evaluate the chronic effects (24-hour-incubation) of berry extract on glucose uptake in differentiated C2C12 muscle cells under basal or insulin-stimulated conditions, with the use of [^{3}H]-labelled D-glucose;
5. To evaluate the chronic effects (24-hour-incubation) of berry extract and insulin on the gene expression of glucose transporters in C2C12 muscle cells, measured by qRT-PCR;
6. To investigate the changes in global gene expression in C2C12 muscle cells in response to 24-hour-incubation with berry extract. This will be carried out using Affymetrix Mouse Genome 430A microarrays;
7. To evaluate the chronic effects (24-hour-incubation) of ethanol on the gene expression of glucose transporters in C2C12 muscle cells, by qRT-PCR;
8. To investigate the changes in global gene expression in C2C12 muscle cells in response to 24-hour-incubation with ethanol, using Affymetrix Mouse Genome 430A microarrays and validated by qRT-PCR.

There was a final objective, namely to investigate if the berry extract, or its polyphenolic components, could act as a myoprotectant, especially in the presence

of ethanol. However, lack of time precluded such studies being carried out within the time of a 4-year PhD programme.

2 Materials and methods

2.1 Cell culture

2.1.1 Caco-2

The TC7 sub-clone of the human intestinal adenocarcinoma cell line, Caco-2, was obtained from Dr Monique Rousset and Dr Edith Brot-Laroche (INSERM U505, Paris, France). Caco-2 cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM, 4500 mg/l glucose) (Sigma, UK), supplemented with 10% (v/v) fetal bovine serum (FBS) (Sigma, UK), 1% (v/v) L-glutamine (Invitrogen, UK), 1% (v/v) MEM non-essential amino acids (NEAAs) (Invitrogen, UK), 50 units/ml penicillin and 50 μg/ml streptomycin (Sigma, UK) and 5 μg/ml plasmocin (Source BioScience, UK), in a humidified atmosphere containing 5% CO_2 at 37˚C. Cell culture medium was changed three times a week. Caco-2 cells were used between passages 40-50. Cells were sub-cultured at confluence by incubating with TrypLE™ Express (Invitrogen, UK) for 15 minutes at 37˚C. For experiments, Caco-2 cells were seeded at a density of 10,000 cells/cm^2 and used 14 days after seeding unless otherwise specified.

2.1.2 C2C12

The murine myoblast cell line, C2C12, was purchased from LGC Standards (UK). Cells were maintained in DMEM (4500 mg/l glucose) (Sigma, UK), supplemented with 10% (v/v) FBS (Sigma, UK), 1% (v/v) L-glutamine (Invitrogen, UK), 1% (v/v) MEM NEAAs (Invitrogen, UK), 50 units/ml penicillin and 50 μg/ml streptomycin (Sigma, UK) and 5 μg/ml plasmocin (Source BioScience, UK), in a humidified atmosphere containing 5% CO_2 at 37˚C. Cell culture medium was changed two to three times a week. Differentiation of myoblasts into myotubes was induced at 90-100% confluency by replacing FBS with 10% (v/v) horse serum (Sigma, UK). C2C12 myotubes were used between passages 4-14. Cells were sub-cultured at 80% confluence by incubating

with TrypLE™ Express (Invitrogen, UK) for 15 minutes at 37˚C. For experiments, C2C12 cells were seeded at 5,000 cells /cm^2 and used 7 days after induction of differentiation unless otherwise specified.

2.2 Berry extract and polyphenols

The berry extract (OptiBerry®, InterHealth Nutraceuticals; USA) used in this study is derived from a unique blend of wild blueberry, strawberry, cranberry, wild bilberry, elderberry and raspberry prepared by ethanol extraction. It is sugar-free and does not have detectable vitamin C. **Table 6** shows a typical analysis of the berry extract provided by the manufacturer. Attempts were made to investigate its composition in more detail using nuclear magnetic resonance (NMR) spectroscopy, but lack of time precluded this (see Section 6 on Future Investigations). Since OptiBerry® is a commercialised product, no other information is available. The berry extract was stored in an air-tight container at -20˚C.

Table 6. Typical analysis of berry extract

Analysis	Value
ORAC[1] (μmol TE[2]/g)	≥3700
Polyphenolic content (mg GAE[3]/g)	≥200
Total anthocyanins content (mg/g)	≥80
Malvidin (mg/g)	≥8.0
Cyanidin (mg/g)	≥30
Delphinidin (mg/g)	≥30
Petunidin (mg/g)	≥8.0
pH (1g/100 ml in water)	2.5-3.5

[1]ORAC: Oxygen Radical Absorbance Capacity
[2]TE: Trolox equivalent
[3]GAE: Gallic acid equivalent
Analysis was performed by InterHealth Neutraceuticals, USA.

All other polyphenols, cyanidin chloride, cyanidin-3-*O*-glucoside chloride, cyanidin-3-*O*-rutinoside chloride, phloretin, phloridzin, quercetin and quercetin-3-*O*-glucoside, were purchased from Sigma (UK).

2.3 Quantitative real time-polymerase chain reaction (qRT-PCR)

2.3.1 RNA extraction

Total RNA was isolated from cells using TRIzol® reagent (Invitrogen, UK) according to manufacturer's instructions described as follows. Cultured cells were homogenised in TRIzol® reagent directly in the culture dish by pipetting up and down several times. Homogenised cells were transferred to a centrifuge tube followed by the addition of chloroform. After being subject to vigorous shaking and centrifugation at 12,000 *g* at 4˚C, RNA was then isolated in the aqueous phase by phase separation and precipitated with ice-cold isopropanol. The resultant RNA pellet recovered after another centrifugation at 12,000 *g* at 4˚C was further washed with ethanol.

2.3.2 Reverse transcription

Extracted RNA pellet was re-suspended in nuclease-free water and reverse transcribed into cDNA using High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, UK), which contains the MultiScribe™ reverse transcriptase, dNTP, random primers and RNase inhibitor, according to manufacturer's protocol, on MJ Research PTC-225 Peltier Thermo Cycler (Labtech, UK). Thermal cycling conditions for reverse transcription are shown in **Table 7**.

Table 7. Thermal cycling conditions for reverse transcription (High Capacity cDNA Reverse Transcription Kit)

Step	Primer binding	Reverse transcription	Enzyme inactivation	Cooling
Temperature	25˚C	37˚C	85˚C	4˚C
Time	10 min	120 min	5 sec	∞

2.3.3 qRT-PCR

Specific primers for qRT-PCR were designed using the Primer-BLAST primer designing tool (http://www.ncbi.nlm.nih.gov/tools/primer-blast/). PCR product sizes were restricted to between 60 and 300 base pairs. **Table 8** and **Table 9** show the primer sequences of the genes tested and their amplicon sizes.

qRT-PCR was performed using either SYBR® Green PCR Master Mix (Applied Biosystems, UK), which contains SYBR® Green I Dye, AmpliTaq® Gold DNA Polymerase and dNTPs with dUTP, or Fast SYBR® Green Master Mix (Invitrogen, UK), which contains SYBR® Green I Dye, AmpliTaq® Fast DNA Polymerase and dNTPs, and custom-made primers (Eurofins MWG Operon, Germany) at a final concentration of 400 nM. Thermal cycling conditions for qRT-PCR are shown in **Table 10** and **Table 11**. Typical PCR standard curve and dissociation curves are shown in **Figure 45**, page 224 and **Figure 46**, page 225 in the Appendix. DNA agarose gel electrophoresis was performed to confirm the sizes of PCR products. **Figure 47** (page 225, in the Appendix) shows typical images of DNA agarose gel electrophoresis.

To analyse the relative changes in the expression of genes of interest, the delta delta Ct method was used (Livak and Schmittgen, 2001). In this method, the PCR signals of the target transcript were normalised to at least one housekeeping gene. The changes in expression were subsequently quantified in relation to the values of the respective

control treatment. In Caco-2 cells, at least one of the following stably-expressed housekeeping genes were used for normalisation- 18S ribosomal RNA, beta-actin, beta-2 microglobulin, glyceraldehyde 3-phosphate dehydrogenase and beta-tubulin 2C. In C2C12 cells, normalisation was performed using glyceraldehyde 3-phosphate dehydrogenase and/or hypoxanthine phosphoribosyltransferase 1. Selection was based on a number of criteria, which included their abundance, their stability and how common they were used by other groups in the literature.

Table 8. List of primer sequences (*Homo sapiens*)

Gene symbol*	Forward primer	Reverse primer	Amplicon length (bp)
18S	AACTTTCGATGGTAGTCGCCG	CCTTGGATGTGGTAGCCGTTT	105
ACTB	TCCTTCCTGGGCATGGAGT	GCACTGTGTTGGCGTACAG	103
B2M	CCACTGAAAAAGATGAGTATGCCT	CCAATCCAAATGCGGCATCTTCA	126
GAPDH	ACCCACTCCTCCACCTTTGA	CTGTTGCTGTAGCCAAATTCGT	101
TUBB2C	TTGGGAGGTGATCAGCGATGAG	CTCCAGATCCACGAGCACGGC	148
SLC2A1	CCAGCTGCCATTGCCGTT	GACGTAGGGACCACACAGTTGC	99
SLC2A2	AGTTAGATGAGGAAGTCAAAGCAA	TAGGCTGTCGGTAGCTGG	165
SLC2A3	CAATGCTCCTGAGAAGATCATAA	AAAGCGGTTGACGAAGAGT	172
SLC2A5	CACCCACTTACTTAGCCAAAC	GCACTGAATGGAGAGAGAAGAC	169
SLC5A1	CGCCTATCCAACCTTAGTGGTG	CGCTGTTGAAGATGGAGGTCAG	110
SI	CATCCTACCATGTCAAGAGCCA	GCTTGTTAAGGTGGTCTGGTTT	196

* Listed genes encoded the following products: *18S*- 18S ribosomal RNA; *ACTB*- beta-actin; *B2M*- beta-2 microglobulin; *GAPDH*- glyceraldehyde 3-phosphate dehydrogenase; *TUBB2C*- beta-tubulin 2C; *SLC2A1*- glucose transporter 1 (GLUT1); *SLC2A2*- glucose transporter 2 (GLUT2); *SLC2A3*- glucose transporter 3 (GLUT3); *SLC2A5*- glucose transporter 5 (GLUT5); *SLC5A1*- sodium/glucose co-transporter 1 (SGLT1); *SI*- sucrose-isomaltase.

Table 9. List of primer sequences (*Mus musculus*)

Gene symbol*	Forward primer	Reverse primer	Amplicon length (bp)
GAPDH	ATGACCACAGTCCATGCCATC	CCTGCTTCACCACCTTCTTG	271
HPRT1	GCAAACTTTGCTTTCCCTGG	ACTTCGAGAGGTCCTTTTCACC	85
SLC2A1	CTTCCTGCTCATCAATCGT	AGCTCCAAGATGGTGACCTT	150
SLC2A4	TGCTCTCCTGCAGCTGATT	TTCAGCTCAGCTAGTGCGTC	147

* Listed genes encoded the following products: *GAPDH*- glyceraldehyde 3-phosphate dehydrogenase; *HPRT1*- hypoxanthine phosphoribosyltransferase 1; *SLC2A1*- glucose transporter 1 (GLUT1); *SLC2A4*- glucose transporter 4 (GLUT4).

Table 10. Thermal cycling conditions for qRT-PCR (SYBR® Green PCR Master Mix)

Step	Enzyme activation	PCR	
		Denature	Anneal/Extend
	Hold	Cycle (40 cycles)	
Temperature	95˚C	95˚C	60˚C
Time	10 min	15 sec	1 min

Table 11. Thermal cycling conditions for qRT-PCR (Fast SYBR® Green Master Mix)

Step	Enzyme activation	PCR	
		Denature	Anneal/Extend
	Hold	Cycle (40 cycles)	
Temperature	95˚C	95˚C	60˚C
Time	20 sec	3 sec	30 sec

2.4 Trypan blue exclusion viability test

Trypan blue is a diazo dye that does not pass through cell membrane. Cells with intact membrane are devoid of staining by trypan blue. It therefore distinguishes living cells from dead cells by the presence of intact membrane.

Adhered cells were washed with phosphate buffered saline (PBS) twice before being subject to dissociation with TrypLE™ Express (Invitrogen, UK) at 37˚C for 15 minutes. Normal cell culture medium was added to harvest the cells. The cell suspension was centrifuged at 600 rpm for 5 minutes using the Eppendorf centrifuge 5804R (Eppendorf, UK). After decanting the supernatant, the cell pellet was re-suspended in cell culture medium. For each millilitre of cell suspension, 0.1 ml of 0.4% trypan blue solution (Sigma, UK) was added and loaded onto a hemocytometer, and examined under Nikon Eclipse TS100 inverted microscope (Nikon, UK) at 100 times magnification. The numbers of total cells and blue-staining cells were counted and percentage of viable cells was calculated as followed:

% viable cells = [1.00 – (Number of blue cells ÷ Number of total cells)] × 100%

2.5 MTS viability assay

MTS (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium) is a yellow tetrazolium compound. In metabolically active cells, the NADPH or NADH generated by dehydrogenase enzymes reduce MTS into a purple-coloured formazan product that has an absorbance maximum at 490 nm (Berridge and Tan, 1993). **Figure 9** shows the structures of MTS and its formazan product.

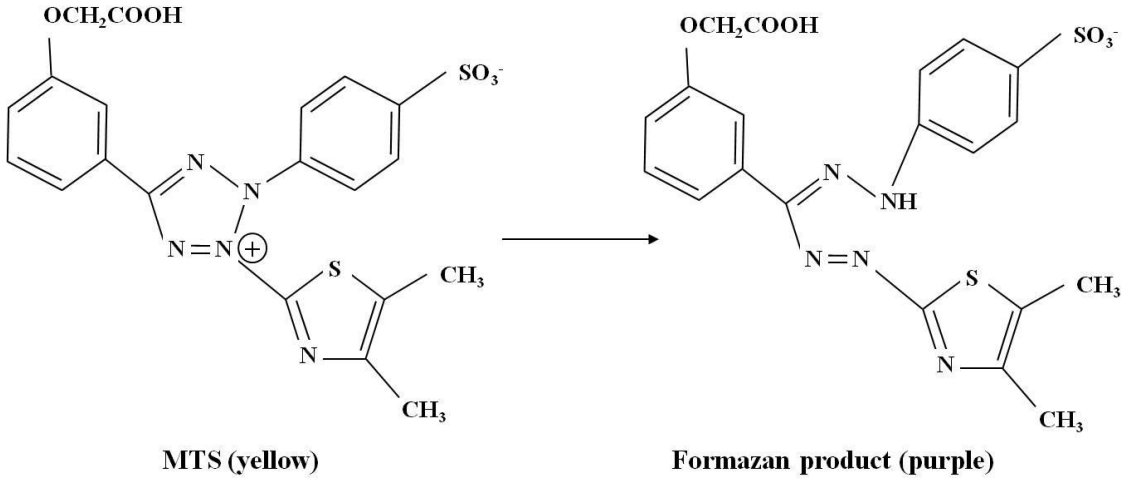

Figure 9. Reduction of MTS tetrazolium into formazan product
Adapted from the instruction manual of the CellTiter 96® AQueous One Solution Cell Proliferation Assay.

MTS viability assay was performed using CellTiter 96® AQueous One Solution Cell Proliferation Assay (Promega, UK). Briefly, C2C12 cells were seeded on 96-well plates at a density of 5,000 cells/cm^2. After treatment with berry extract, incubating solutions were changed to 100 µl cell culture medium, followed by addition of 20 µl of CellTiter 96® AQueous One Solution Reagent. After incubation at 37˚C for 45 minutes, absorbance at 490 nm was read using BioTek Synergy HT Multidetection Microplate Reader (Fisher Scientific, UK).

2.6 Giemsa staining

Differentiation of C2C12 myoblasts into myotubes was monitored by Giemsa staining (Freshney, 2010). Giemsa stain (BDH, UK) stains the nuclei pink, the chromosomes dark purple, the cytoplasm pale grey-pink and the protein-rich myotubes dark red-purple.

Staining was started by washing adherent cells with PBS after discarding the cell culture medium. The cells were fixed in PBS/methanol (1:1) for 2 minutes followed by another 10 minutes in fresh anhydrous methanol. After a brief rinse with fresh methanol, the cells were either dried and stored, or stained directly. To stain, neat Giemsa stain was added to cover the cell monolayer for 2 minutes. Water was added to dilute the stain with agitation of the dish gently for further 2 minutes. The stain was then displaced upward and washed under running tap water until any pink cloudy background stain was removed. The monolayer was finally rinsed in deionised water. Stained cells were examined using Nikon Eclipse TE200 microscope (Nikon, UK) attached to Nikon digital camera DXM 1200 (Nikon, UK).

2.7 Global gene expression studies

Global gene expression studies with the use of microarrays were performed with the assistance on equipment from technicians, Dr Matthew Arno, Dr Estibaliz Aldecoa-Otalora Astarloa and Dr Yuen Fei Wong, in the Genomics Centre, Waterloo Campus, King's College London. Otherwise, samples were handled by the PhD candidate.

2.7.1 Ethanol precipitation for RNA purification

To purify RNA samples for microarray studies, sodium acetate (pH 5.2) was added to the RNA samples dissolved in nuclease-free water. Subsequently, ice-cold 100%

ethanol was added, mixed thoroughly with the RNA and kept at -20°C overnight. The precipitated RNA was separated by centrifugation at 13,000 *g* at 4°C and further washed with ice-cold ethanol. The resultant purified RNA pellet was then dissolved in nuclease-free water for subsequent use. RNA integrity was checked using Agilent RNA 6000 Nano Kit on the 2100 Agilent Bioanalyzer Instrument (Agilent, UK). **Figure 10** shows a typical RNA integrity report for our RNA samples.

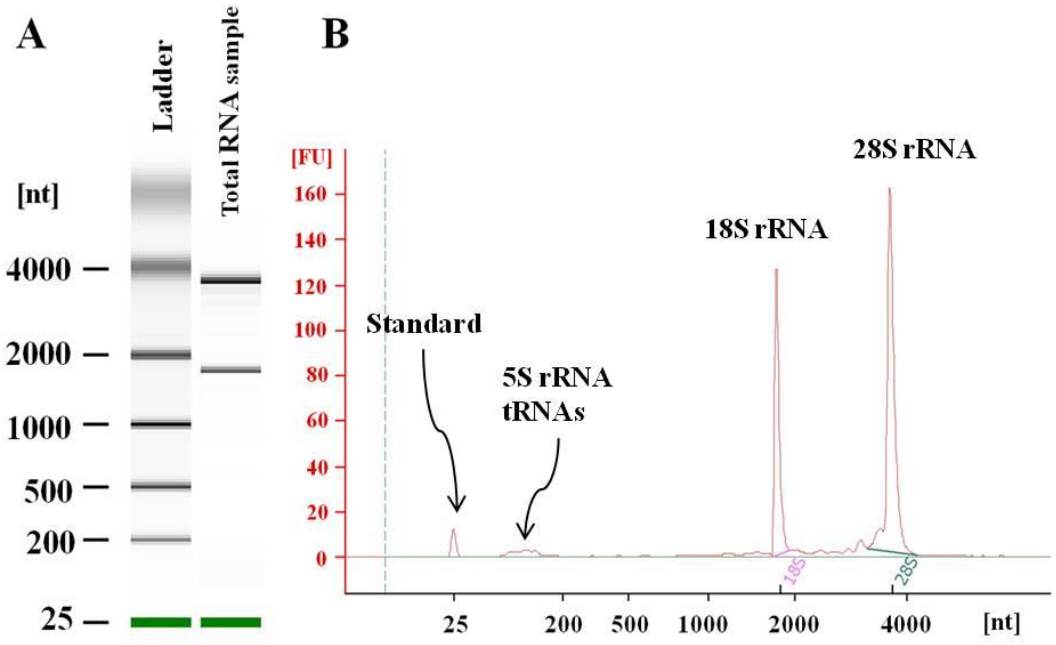

Figure 10. Typical Agilent Bioanalyzer report of total RNA samples
Figures shown are gel-like image (A) and electropherogram (B) of total RNA samples. The 18S and 28S rRNAs appeared as peaks at around 1800 and 4000 nt respectively. The RNA integrity numbers (RIN) of all RNA samples used are between 9.80 and 10.0.

2.7.2 RNA amplification and labelling

Purified RNA samples were pooled and 500 ng of each pooled sample in 5 µl was used in the reactions. Reverse transcription to synthesise first strand cDNA, second strand cDNA synthesis and *in vitro* transcription (IVT) to synthesise biotin-modified aRNA were carried out using MessageAmp^{TM} Premier RNA Amplification Kit (Invitrogen, UK) according to manufacturer's protocol. **Table 12** shows the thermal cycling

conditions for the above-mentioned reactions. After synthesis, the biotin-modified aRNAs were purified to remove enzymes, salts and unincorporated nucleotides using the same kit.

Table 12. Thermal cycling conditions for the reactions performed using MessageAmp™ Premier RNA Amplification Kit

Reaction	Temperature and time		
First strand cDNA synthesis	42˚C for 2 hr	4˚C indefinite hold	
Second strand cDNA synthesis	16˚C for 1 hr	65˚C for 10 min	4˚C indefinite hold
IVT	40˚C for 14 hr	4˚C indefinite hold	

2.7.3 aRNA fragmentation and hybridisation

Following purification, the biotin-modified aRNAs were subjected to fragmentation using the GeneChip® 3' IVT Express Kit (Affymetrix, UK). Sizes of the fragmentation reaction products were analysed on the Agilent 2100 Bioanalyzer (Agilent, UK) with Agilent RNA 6000 Nano Kit (Agilent, UK). The fragmented aRNAs had a distribution of 35-200 nt with a peak at approximately 100-120 nt. **Figure 11** shows typical Bioanalyzer electropherograms of unfragmented and fragmented cRNAs. Afterwards, 12 μg of aRNAs were hybridised onto the Affymetrix Mouse Genome 430A 2.0 Array (Affymetrix, UK), which is comprised of over 22,600 probe sets representing over 14,500 well-substantiated mouse genes. The arrays were subsequently washed, stained and scanned in the Fluidics Station 400 and the GeneArray Scanner (Affymetrix, UK) according to the Affymetrix recommended protocol.

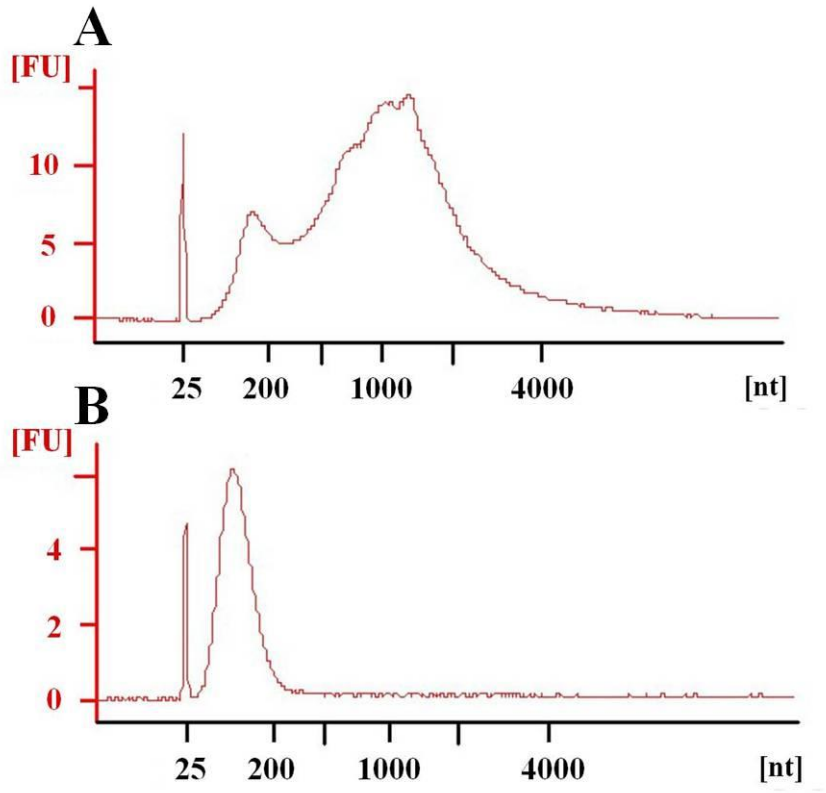

Figure 11. Typical Agilent Bioanalyzer electropherograms of cRNAs
Unfragmented cRNA (**A**) shows a distribution of sizes from 250 to 5500 nt with most of the cRNA between 600 and 1200 nt; fragmented cRNA (**B**) shows a size distribution of 35–200 nt with a peak at approximately 100-120 nt.

2.7.4 Microarray data analysis

Microarray Suite version 5.0 (MAS 5.0) algorithm was used for normalisation and generation of gene expression summaries from the array images. The MAS 5.0 algorithm normalises data at probe set level using linear scaling method. It generates detection calls that indicate whether the target sequence is present (P), marginally present (M) or absent (A) in the given sample by comparing the signal intensities from perfect-match probes to their corresponding mismatch probes in a specific probe set. In the present study, targets that were either called as P or M in at least one group were included for analysis.

2.7.5 Gene functional classification

Altered gene lists were imported into the bioinformatics resource, the Database for Annotation, Visualisation and Integrated Discovery (DAVID) version 6.7 (http://david.abcc.ncifcrf.gov/home.jsp) (National Institute of Allergy and Infectious Diseases, NIH). Using the gene functional classification tool, as well as our own literature search, the differentially expressed genes were classified into functionally-related gene clusters.

2.7.6 Validation by qRT-PCR

Validation of microarray results by qRT-PCR was performed using TaqMan® Universal PCR Master Mix (Applied Biosystems, UK), which contains the AmpliTaq® Gold DNA polymerase and dNTPs with dUTP. Universal ProbeLibrary (UPL) probes, which are labelled at the 5' end with fluorescein (FAM) and at the 3' end with a dark quencher dye, and target-specific PCR primers were designed using the ProbeFinder software available online at the Universal ProbeLibrary Assay Design Center (https://www.roche-applied-science.com) (see **Table 13** for primer sequences and probe identities). The probes were purchased from Roche Diagnostics (UK) and used at a final concentration of 100 nM. The primers were obtained from Eurofins MWG Operon (Germany) and used at a final concentration of 200 nM. Thermal cycling conditions for TaqMan qRT-PCR are shown in **Table 14**. The delta delta Ct method was used to analyse the relative changes in gene expression. Glyceraldehyde 3-phosphate dehydrogenase and hypoxanthine phosphoribosyltransferase 1 were stably expressed and selected as housekeeping genes.

Table 13. List of PCR primer sequences and UPL probes for validation of microarray results

Gene name*	Forward primer	Reverse primer	UPL probe number	Amplicon size (nt)
AKT2	CGACCCAACACCTTTGTCA	GATAGCCCGCATCCACTCT	#27	105
ANKRD2	CGGACACCTGTGATGAGTTC	GCCCCATTCTCCAGAAGTTT	#56	94
GAPDH	GGGTTCCTATAAATACGGACTGC	CCATTTTGTCTACGGGACGA	#52	112
HPRT1	TCCTCCTCAGACCGCTTTT	CCTGGTTCATCATCGCTAATC	#90	95
INSR	TCTTTCTTCAGGAAGCTACATCTG	TGTCCAAGGCATAAAAAGAATAGTT	#91	76
IRS2	TCCAGGCACTGGAGCTTT	GGCTGGTAGCGCTTCACT	#53	107
MYH2	AAAGCTCCAAGGACCCTCTT	AGCTCATGACTGCTGAACTCAC	#33	81
MYH7	CGCATCAAGGAGCTCACC	CTGCAGCCGCAGTAGGTT	#6	60
PIK3CB	GCGGAGACAGTGCTAATGTG	CCCTGTCCAAGATTTCTTTCAG	#31	75
TBC1D1	AACTCATGCGGTACCACTCC	ACCCCCAGGAAGATGGTC	#99	71
TNNI3	GCAGGTGAAGAAGGAGGACA	CGATATTCTTGCGCCAGTC	#10	65
TPM1A	GTCATCATCGAGAGCGACCT	TCTTCAAGCTCGGCACATTT	#82	77

* Listed genes encoded the following products: *AKT2*- V-akt murine thymoma viral oncogene homolog 2; *ANKRD2*- Ankyrin repeat domain-containing protein 2; *GAPDH*- glyceraldehyde 3-phosphate dehydrogenase; *HPRT1*- hypoxanthine phosphoribosyltransferase 1; *INSR*- insulin receptor; *IRS2*- insulin receptor substrate 2; *MYH2*- myosin heavy chain IIa; *MYH7*- myosin heavy chain β; *PIK3CB*- phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit beta isoform; *TBC1D1*- TBC1 (tre-2/USP6, BUB2, cdc16) domain family, member 1; *TNNI3*- troponin I, type 3 and *TPM1A*- tropomyosin 1 alpha.

Table 14. Thermal cycling conditions for qRT-PCR (TaqMan® Universal PCR Master Mix)

Step	UNG * incubation	AmpliTaq Gold activation	PCR	
	Hold	Hold	Cycle (40 cycles)	
			Denature	Anneal/ extend
Temperature	50˚C	95˚C	95˚C	60˚C
Time	2 min	10 min	15 sec	1 min

*UNG: AmpErase® uracil-N-glycosylase, a component of the TaqMan® Universal PCR Master Mix that can prevent the re-amplification of carryover-PCR products by removing any uracil incorporated into single- or double-stranded DNA (Longo et al., 1990).

2.8 MicroRNA studies

2.8.1 MicroRNA expression profiling

Total RNA was extracted using TRIzol® reagent (Invitrogen, UK) according to manufacturer's protocol, except for an additional overnight precipitation step at -20°C during isopropanol incubation. Extracted RNA was labelled using Genisphere® FlashTag™ Biotin HSR RNA Labeling Kit (Affymetrix, UK) and miRNA profiling was performed using the GeneChip® miRNA array (Affymetrix, UK), which contains 847 specific probe sets for human miRNAs derived from the Sanger miRBase miRNA database v11. Robust multiarray average algorithm (RMA) was used for summarising probe level data, by quantile normalisation. miRNA target prediction was performed using the bioinformatics algorithm, *TargetScanHuman version 6.2* (http://www.targetscan.org/).

2.8.2 Validation by qRT-PCR

Validation of miRNA expressions of five selected miRNAs (*let-7a, let-7d, let-7f, miR-92b* and *miR-106b*) was performed according to miRCURY LNA™ Universal RT microRNA PCR instruction manual. Extracted RNA (10 ng) was reverse transcribed using the Universal cDNA synthesis kit (Exiqon, Denmark) and qRT-PCR was performed using commercially available miRNA-specific primers and SYBR® Green master mix (Exiqon, Denmark). Thermal cycling conditions for reverse transcription and qRT-PCR are shown in **Table 15** and **Table 16**. **Figure 12** shows the schematic outline of the principles of miRCURY LNA™ Universal RT microRNA PCR system. Analysis of PCR data was carried out using delta delta Ct method with two stably expressed miRNAs- *miR-19b* and *miR-200a*- as housekeeping genes.

Table 15. Thermal cycling conditions for reverse transcription (miRCURY LNA™ Universal RT microRNA PCR kit)

	Reverse transcription	Enzyme inactivation	Cooling
Temperature	42˚C	95˚C	4˚C
Time	60 min	5 min	∞

Table 16. Thermal cycling conditions for qRT-PCR (miRCURY LNA™ Universal RT microRNA PCR kit)

Step	**Enzyme activation**	**PCR**	
		Denature	**Anneal/Extend**
	Hold	**Cycle (40 cycles)**	
Temperature	95˚C	95˚C	60˚C
Time	10 min	10 sec	1 min

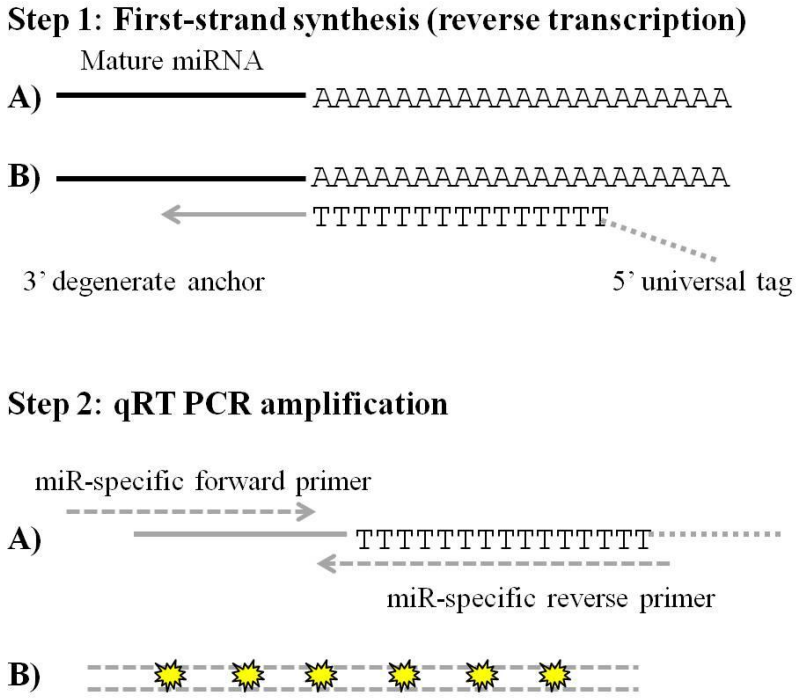

Figure 12. Schematic outline of the miRCURY LNA™ Universal RT microRNA PCR system

A poly-A tail is added to the mature microRNA template (step **1A**). cDNA is synthesised using a poly-T primer with a 3' degenerate anchor and a 5' universal tag (step **1B**). The cDNA template is then amplified using microRNA-specific and LNA™-enhanced forward and reverse primers (step **2A**). SYBR® Green is used for detection (step **2B**). Reproduced from miRCURY LNA™ Universal RT microRNA PCR instruction manual.

2.9 Uptake studies

Sugar uptake assays were carried out in HEPES-buffered salt solution (pH 7.4; 130 mM NaCl, 4 mM KH_2PO_4, 1 mM $MgSO_4$, 1 mM $CaCl_2$, 30 mM HEPES, and 0.2% (w/v) bovine serum albumin) with D-glucose and 0.15 μCi/ml D-[6-^{3}H]-glucose (PerkinElmer, UK), or D-fructose and 0.15 μCi/ml D-[^{14}C]-fructose (American Radiolabeled Chemicals, UK).

Caco-2 cells or C2C12 myotubes were incubated for 15 minutes at room temperature in HEPES buffer prior to experiments. Uptake was initiated by the addition of either the control or test solutions and was terminated after 2 minutes by aspiration of the uptake buffer, immediately followed by the addition of ice-cold PBS. Rate of glucose uptake at 2 minutes at room temperature has been demonstrated previously by our research group to be linear (unpublished data, Dr Paul Sharp). Thus, it was considered prudent to determine the influence of analytes during the linear kinetic phase of glucose uptake. Cells were washed twice more with ice-cold PBS and solubilised at 37˚C in 0.2% (w/v) sodium dodecyl sulfate (SDS) for 1 hour prior to scintillation counting (LS 6500, Beckman Coulter, UK). Aliquots of cell lysate were taken to determine protein content by Bradford protein assay (Bradford, 1976).

2.10 Statistical analyses

All data are expressed as mean ± standard error of mean (SEM) and tested for outliers by Grubb's test using the free web calculator provided by GraphPad Software (http://graphpad.com/quickcalcs/Grubbs1.cfm). Sample sizes are described in the legend to each figure. Independent-sample t-test was used for two group comparisons. For multiple group comparisons, data were first tested for normality using Shapiro-Wilk test. In case of significant deviation from normal distribution, data were analysed using

the non-parametric independent-samples Kruskal-Wallis test with Mann-Whitney test. Otherwise, data analyses were performed using one-way ANOVA with Dunnett's or Tukey post-hoc tests. In all studies, statistical significance was reached when P-value is less than an alpha level of 0.05.

3 Results

3.1 Intestinal Caco-2 cells

3.1.1 Characterisation of the intestinal Caco-2 cells

3.1.1.1 Morphology

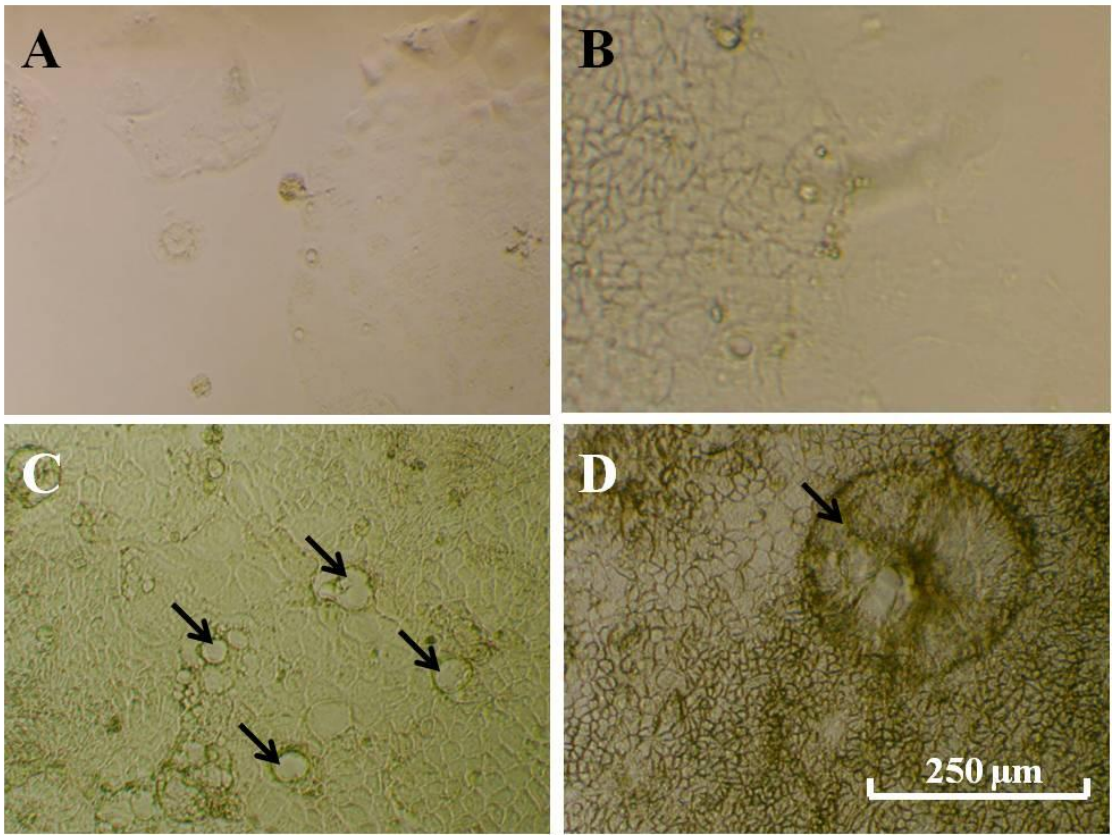

Figure 13. Morphological changes of Caco-2 cells during cell culture

The day of seeding was regarded as Day 0. Images were taken at Day 3 (**A**); Day 7 (**B**); Day 10 (**C**); Day 14 (**D**). The Caco-2 cell monolayer reached 100% confluence at Day 10 (determined by personal observation). Arrows indicate location of domes (100X magnification).

Caco-2 cells were used at passages 40 to 50 and were seeded at a density of 10,000 cells/ cm^2 on plastic culture dishes. Cells attached on the plastic surface within one hour after seeding and appeared as round and flattened cells (not shown). As the cells continued to grow in the following week, they became colloidal in shape and formed clusters by attaching to adjacent cells (**Figure 13A** and **B**). By Day 10 (**Figure 13C**), confluence was reached and small domes were observed on the cell monolayer. The

formation of domes is the consequence of secretion of solutes and water through the basolateral membrane. This suggested there was development of tight junctions between neighbouring cells and ion transport activities, indicating cell differentiation (Grasset et al., 1984). During the culture period after reaching confluence, the number of domes rose progressively and, eventually, small domes fused to form larger domes (**Figure 13D**). Multilayers of cells appeared and cells increased in height, becoming increasingly columnar in shape (personal observation).

3.1.1.2 Gene expression

Relative gene expressions of glucose transporters, GLUT1-3, 5 and SGLT1, as well as the differentiation marker, sucrase-isomaltase, were investigated at several time points during cell culture. In order to represent the three phases of cell growth, i.e. proliferating, confluent and differentiated, three time points (Days 7, 10 and 14) were chosen for analysis.

As shown in **Figure 14**, the gene expression of the differentiation marker, sucrase-isomaltase (SI) increased dramatically from Day 7 to Day 10 by 3.4-fold ($P< 0.005$). There was no further change in the expression of SI after the Caco-2 cells were further cultured for 4 days at Day 14. The relative mRNA levels of GLUT1 and 5 increased progressively from Day 7 to Day 14 ($P< 0.05$) (**Figure 14**). For GLUT2 and 3, the mRNA levels peaked at Day 10 ($P< 0.05$) and decreased thereafter (**Figure 14**). No significant difference was observed in the gene expression of SGLT1 at all three time points, although there was a slight rise from Day 7 to Day 10, which was maintained at Day 14 (**Figure 14**).

In order to compare the relative abundance of mRNAs of the genes investigated, we examined the Ct values (threshold cycle, defined as the number of cycles required for the fluorescent signal to reach the threshold) of those genes from the qRT-PCR. As shown in **Table 17**, at Day 14, the Ct values of GLUT1 and 3 were around 17, whilst the values of GLUT5 and SGLT1 were almost double, i.e. between 30 and 35. The Ct values of GLUT2 and SI were, on the other hand, around 26. As a low Ct value represents higher mRNA level, these results indicated that at Day 14, the mRNA abundances of GLUT1 and 3 were higher than GLUT2 and SI, while those of GLUT5 and SGLT1 were the lowest among all.

Table 17. Ct values of *SLC2A1-3* and *5, SLC5A1* and *SI* in Caco-2 cells at Day 14 obtained from qRT-PCR

Gene name (Encoded protein)	Ct value at Day 14 (Mean ± SEM, n=4)
SLC2A1 (GLUT1)	17.18 ± 0.05
SLC2A2 (GLUT2)	26.85 ± 0.40
SLC2A3 (GLUT3)	17.00 ± 0.05
SLC2A5 (GLUT5)	31.31 ± 0.06
SLC5A1 (SGLT1)	33.98 ± 0.19
SI (sucrase-isomaltase)	26.41 ± 0.11

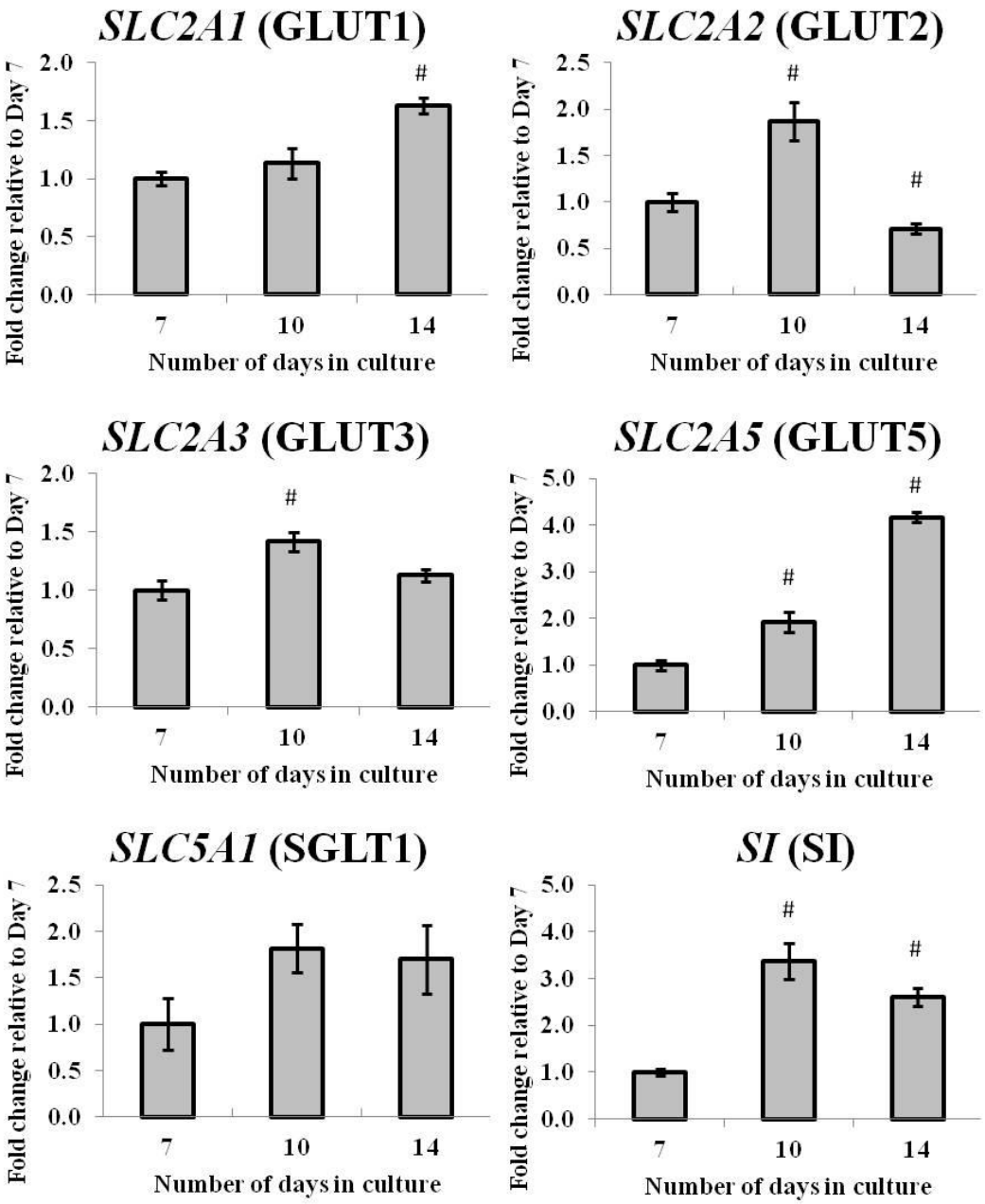

Figure 14. Relative gene expression of *SLC2A1-3* and *5*, *SLC5A1* and *SI* of Caco-2 cells during differentiation

Data are presented as mean ± SEM (n= 4). Gene symbols are indicated above each graph with the encoded proteins in parentheses. $^{\#}P< 0.005$ compared with expression at Day 7.

3.1.1.3 Sugar uptake

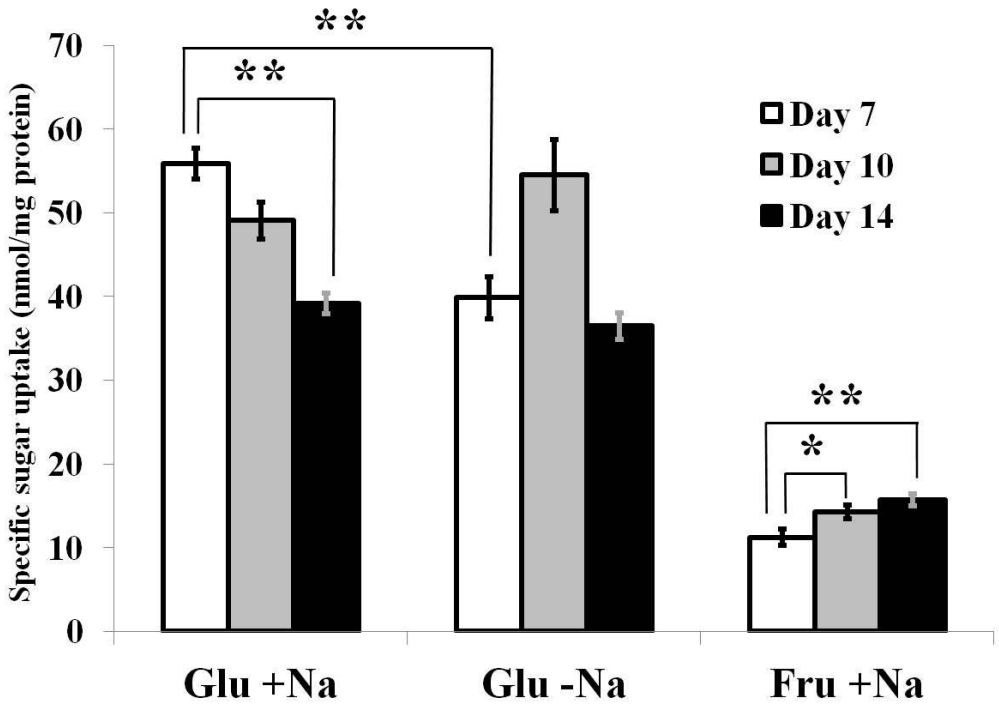

Figure 15. Specific uptake of glucose or fructose by Caco-2 cells cultured for different lengths of time

Uptake of 10 mM glucose with trace of [^{3}H]-labelled D-glucose was measured in the presence (Glu +Na) or absence (Glu –Na) of Na^{+}, and uptake of 10 mM fructose with trace of ^{14}C-labelled fructose was measured in the presence of Na^{+} (Fru +Na). Data are presented as mean ± SEM (n= 6). *P< 0.05, **P< 0.005.

To characterise the Caco-2 cells at the functional level, we investigated the changes in glucose or fructose uptake in Caco-2 cells during differentiation. In the presence of sodium, where uptake occurred via all glucose transporters, i.e. both GLUTs and SGLT1, glucose uptake into Caco-2 cells decreased progressively from Day 7 to Day 14, by around 30% (P< 0.005) (**Figure 15**). Comparing the glucose uptake in the presence and absence of sodium, the latter which favours uptake via GLUTs only, glucose uptake was reduced by around 30% at Day 7 (P< 0.005) (**Figure 15**), when the Caco-2 cells were proliferating. However, there were no significant differences in glucose uptake between the two conditions at Day 10 and Day 14.

Two fructose transporters, GLUT5, which specifically transports fructose only, and GLUT2, which transports both glucose and fructose, are expressed in Caco-2 cells. In the current characterisation experiment, fructose uptake increased progressively during the cell culture period. Compared to Day 7, fructose uptake increased by 27% ($P< 0.05$) and 39% ($P< 0.005$) at Day 10 and Day 14, respectively (**Figure 15**).

3.1.2 Acute effects of berry extract and polyphenols on glucose uptake in the intestinal Caco-2 cells

3.1.2.1 Berry extract

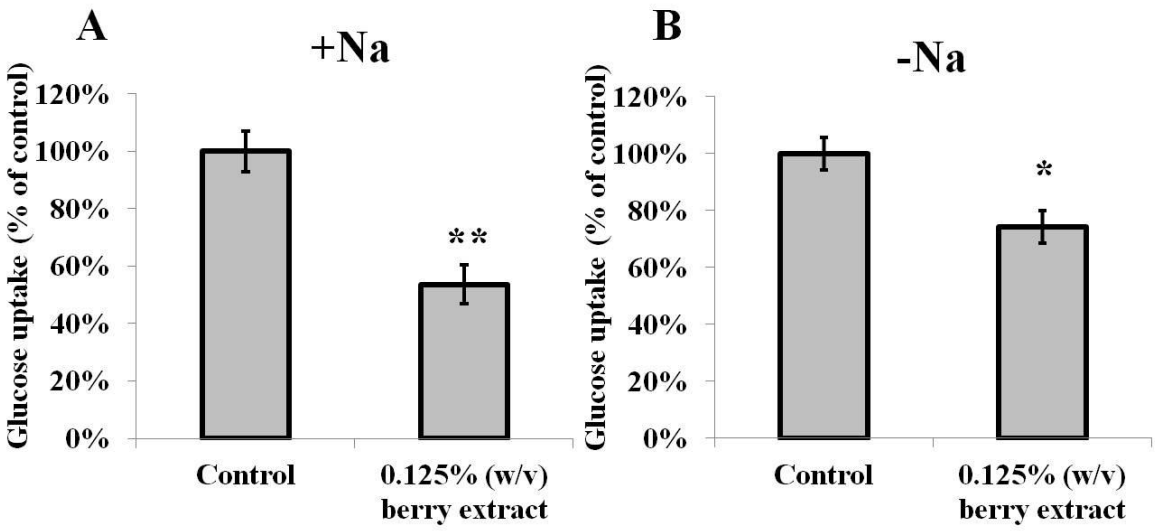

Figure 16. Acute effect of 0.125% (w/v) berry extract on glucose uptake in Caco-2 cells

Uptake of 1 mM D-glucose with trace of [^{3}H]-labelled D-glucose was measured in the presence (**A**) or absence of Na^+ (**B**) after exposure of Caco-2 cells to 0.125% (w/v) berry extract for 15 minutes. Data are presented as mean ± SEM (n= 9). *$P< 0.01$, **$P< 0.001$ compared to respective controls.

Caco-2 cells were exposed acutely to 0.125% (w/v) berry extract for 15 minutes. One millimolar [^{3}H]-D-glucose was then added for another 2 minutes and its uptake was measured. Inhibition of glucose uptake was observed in Caco-2 cells incubated with the berry extract. In the presence of sodium, glucose uptake was reduced by around 50% ($P< 0.001$) (**Figure 16A**), whilst in sodium-free condition which favours transport via

the sodium-independent GLUTs only, a 25% ($P< 0.01$) inhibition was observed (**Figure 16B**).

3.1.2.2 Cyanidin and cyanidin glycosides

The polyphenol analysis report from the manufacturer of the berry extract used in this study showed that cyanidin is one of the most abundant constituent anthocyanidins in the berry extract (≥ 30 mg/g) (see **Table 6**, page 74). In this regard, it is of particular interest whether cyanidin, or its glycosides contribute to the cellular effects of the berry extract on glucose transport in Caco-2 cells. Thus, we investigated the acute effects of the cyanidin aglycone, and two of its glycosides commonly found in natural food, cyanidin-3-*O*-glucoside and cyanidin-3-*O*-rutinoside, on glucose uptake in Caco-2 cells.

Results showed that in sodium-containing buffer, all three tested compounds, i.e. cyanidin, cyanidin-3-*O*-glucoside and cyanidin-3-*O*-rutinoside significantly reduced glucose uptake into Caco-2 cells at a concentration of 100 μM by 27% ($P< 0.005$), 30% ($P< 0.005$) and 16% ($P< 0.05$), respectively (**Figure 17A**). Under sodium-free condition, however, only the glycosides showed inhibitory effect on glucose uptake by 26% ($P< 0.005$) for the glucoside and 21% ($P< 0.05$) for the rutinoside. However, the cyanidin aglycone did not cause any significant change ($P> 0.05$) (**Figure 17B**).

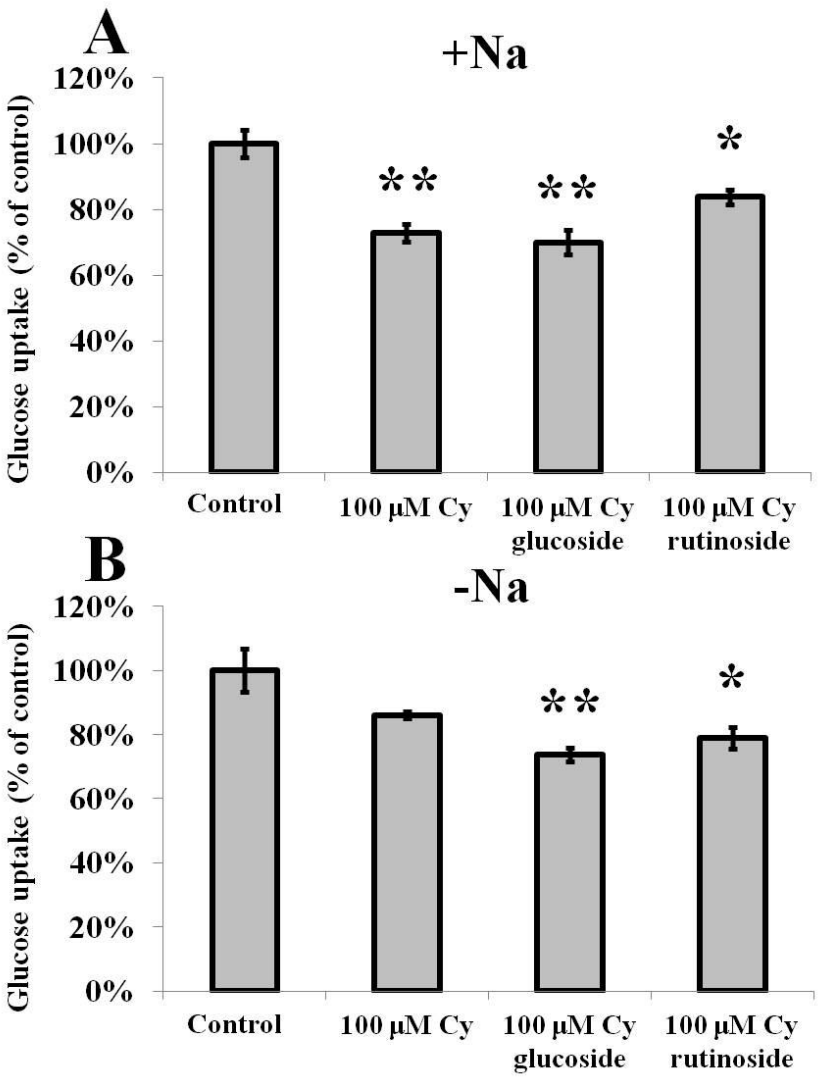

Figure 17. Acute effect of 100 µM cyanidin and its glycosides on glucose uptake in Caco-2 cells

Uptake of 1 mM D-glucose with trace of [^{3}H]-labelled D-glucose was measured in the presence (**A**) or absence of Na^+ (**B**) after exposure of Caco-2 cells to 100 µM of cyanidin (Cy), cyanidin-3-*O*-glucoside (Cy glucoside) or cyanidin-3-*O*-rutinoside (Cy rutinoside) for 15 minutes. Data are presented as mean ± SEM (n= 4). **P*< 0.05, ***P*< 0.005 compared to control.

3.1.2.3 Inhibitors of glucose transporters

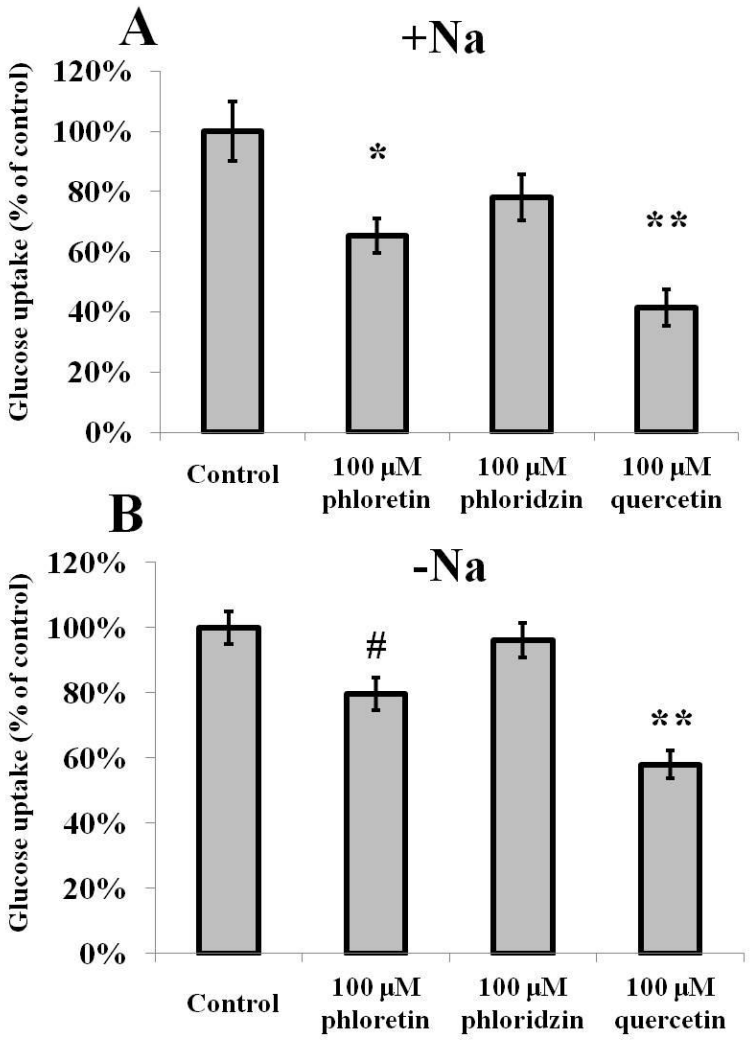

Figure 18. Acute effect of 100 µM of phloretin, phloridzin or quercetin on glucose uptake in Caco-2 cells

Uptake of 1 mM D-glucose with trace of [^{3}H]-labelled D-glucose was measured in the presence (**A**) or absence of Na^+ (**B**) after exposure of Caco-2 cells to 100 µM of phloretin, phloridzin or quercetin for 15 minutes. Data are presented as mean ± SEM (n= 8-9). $^{\#}P< 0.05$, $*P< 0.01$, $**P< 0.001$ compared to control.

We also compared the effects of berry extract on glucose uptake in Caco-2 cells with known inhibitors of glucose transporters, namely phloretin, phloridzin and quercetin. As shown in **Figure 18A** and **B**, 100 µM phloretin inhibited glucose uptake by 35% ($P< 0.01$) and 20% ($P< 0.05$) in the presence and absence of sodium, respectively. At the same concentration, quercetin reduced glucose uptake by 58% ($P< 0.001$) and 42% ($P<$

0.001) in the presence and absence of sodium, respectively. Phloridzin also inhibited glucose uptake by 20% in the presence of sodium albeit not reaching statistical significance ($P > 0.05$).

Investigations of the dose response relationship of the quercetin aglycone and quercetin-3-*O*-glucoside (12.5-200 μM) on glucose uptake revealed that concentrations as low as 12.5 μM exerted a significant inhibitory effect ($P < 0.001$) (**Figure 19A** and **B**). The aglycone (IC_{50} ~37 μM) was a more potent inhibitor than the glucoside (IC_{50} ~55 μM). There was no significant difference between glucose uptake in the presence or absence of sodium for both the aglycone and the glucoside.

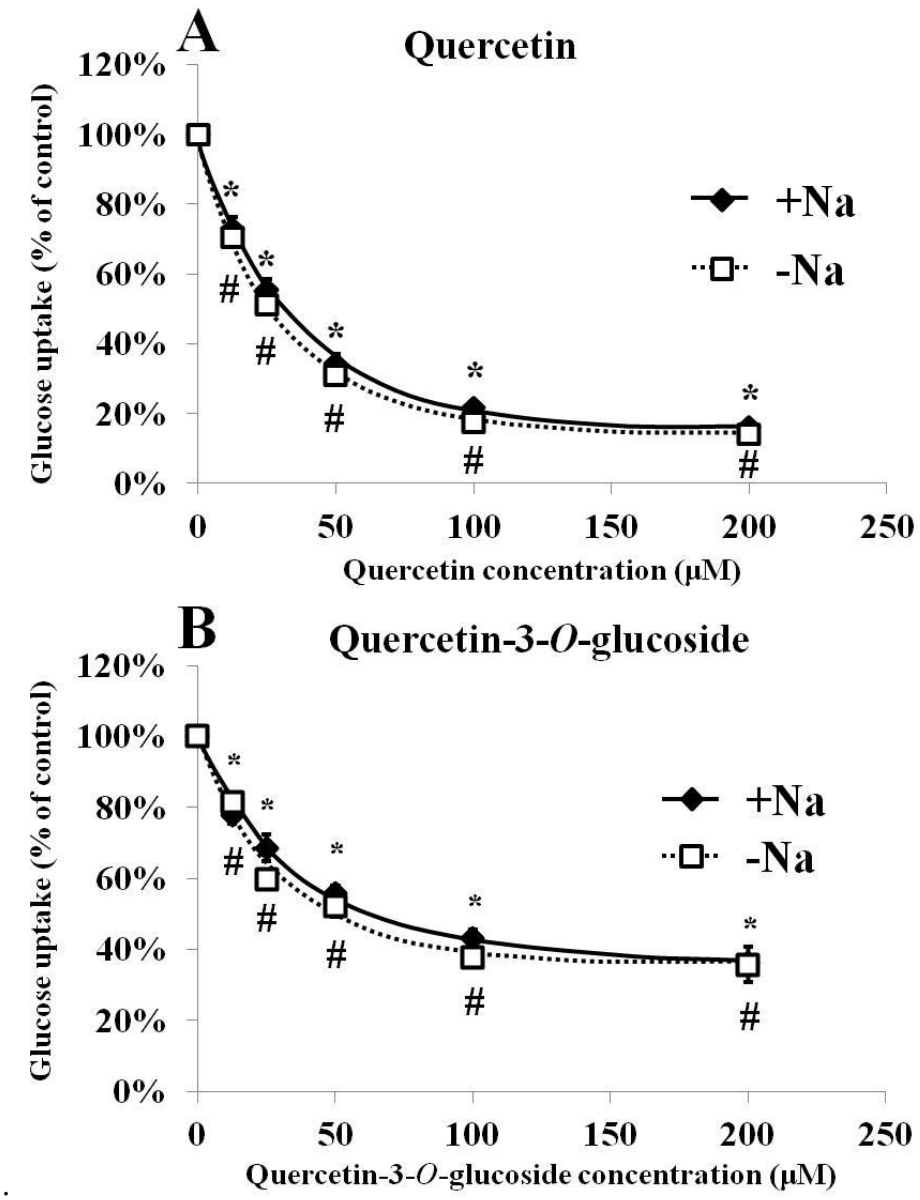

Figure 19. Dose response acute effect of quercetin and quercetin-3-*O*-glucoside on glucose uptake in Caco-2 cells

Uptake of 1 mM D-glucose with trace of [^{3}H]-labelled D-glucose was measured after exposure of Caco-2 cells to 6.25-200 µM of quercetin (**A**) or quercetin-3-*O*-glucoside (**B**) for 15 minutes. Data are presented as mean ± SEM (n= 12). **P*< 0.001 compared to control in the presence of Na^+, $^{\#}P$< 0.001 compared to control in the absence of Na^+.

3.1.3 Chronic effects of berry extract and polyphenols on glucose transport in the intestinal Caco-2 cells

3.1.3.1 Gene expression

Berry extract

In addition to acute treatments (15 minutes), Caco-2 cells were also subjected to an exposure period of 16 hours to investigate the chronic effects on glucose transport.

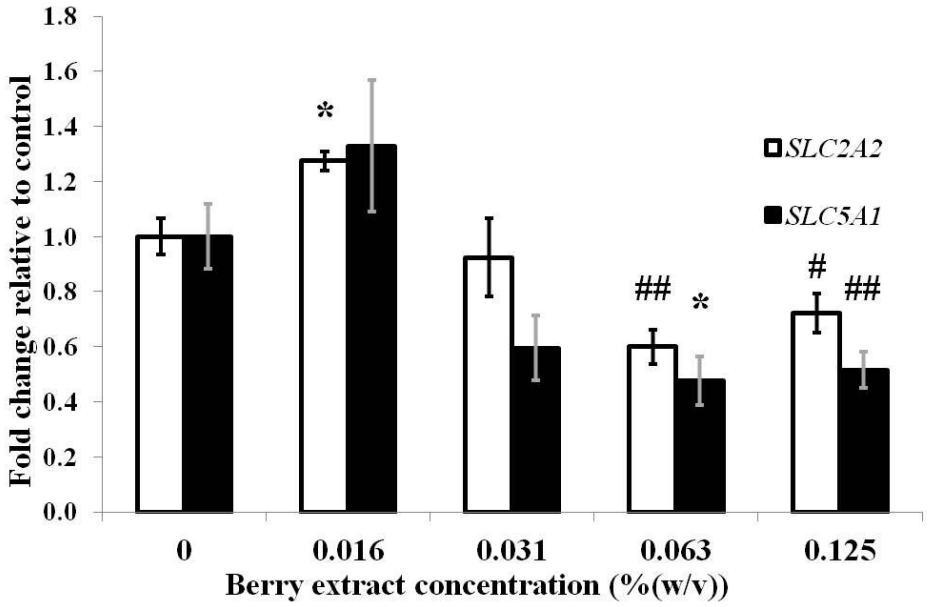

Figure 20. Dose response effect of berry extract on gene expression of *SLC2A2* (GLUT2) and *SLC5A1* (SGLT1) in Caco-2 cells

Caco-2 cells were treated with various concentrations of berry extract (0.016-0.125% (w/v)) for 16 hours. Relative gene expression of *SLC2A2* (GLUT2) and *SLC5A1* (SGLT1) was determined by qRT-PCR. Data are presented as mean ± SEM (n= 8). $^{\#}P< 0.05$, $*P< 0.01$, $^{\#\#}P< 0.005$ compared to respective controls.

Dose response studies demonstrated that berry extract at concentrations ranging from 0.016 to 0.125% (w/v) triggered a biphasic change in the gene expression of GLUT2 (**Figure 20**). At a lower dose of berry extract (0.016% (w/v)), expression of GLUT2 was elevated by 27% ($P< 0.05$), but at higher doses, i.e. 0.063 and 0.125% (w/v), it was reduced by 40% ($P< 0.005$) and 27% ($P< 0.05$), respectively. Significant changes were observed in the gene expression of SGLT1 only at high doses, i.e. 0.063 and 0.125%

(w/v) (**Figure 20**), where the berry extract induced down-regulation by 53% ($P< 0.01$) and 49% ($P< 0.005$), respectively.

In addition to investigating the effects of berry extract on the two major intestinal glucose transporters, GLUT2 and SGLT1, we also investigated the effects on the expression of GLUT1 GLUT3 and GLUT5. GLUT1 and GLUT3 are glucose transporters present in the Caco-2 cell line and GLUT5 is a fructose transporter expressed in human intestinal epithelium. As shown in **Figure 21**, there was no significant change in the expression of GLUT1 across all concentrations of berry extract tested ($P> 0.05$). On the other hand, both GLUT3 and GLUT5 were down-regulated upon treatment with the highest concentration of berry extract at 0.125% (w/v), by 39% ($P< 0.05$) and 76% ($P< 0.01$), respectively (**Figure 21**).

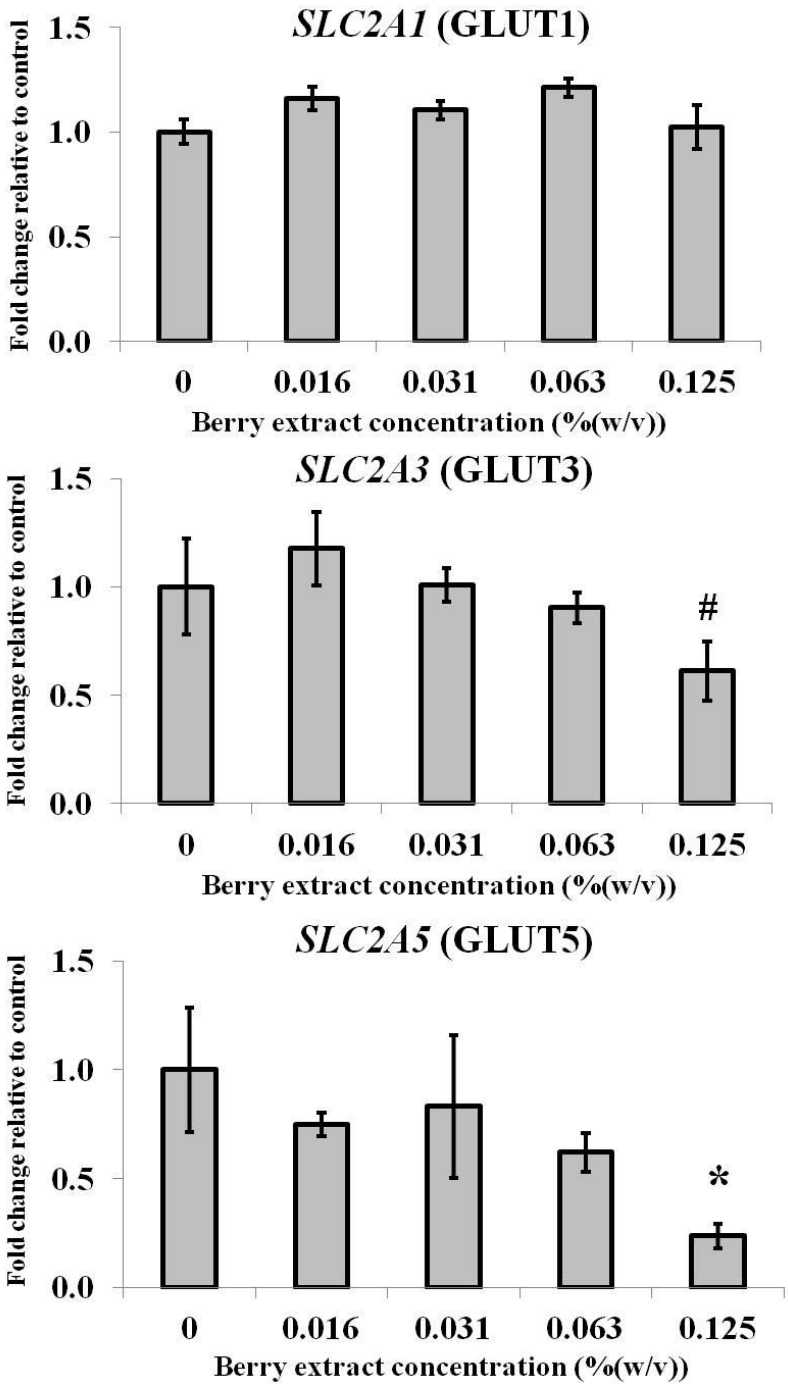

Figure 21. Dose response effect of berry extract on gene expression of *SLC2A1, 3* and *5* (GLUT1, 3, and 5) in Caco-2 cells

Caco-2 cells were treated with various concentrations of berry extract (0.016-0.125% (w/v)) for 16 hours. Relative gene expression of *SLC2A1*, *3* and *5* (GLUT1, 3, and 5) was determined by qRT-PCR. Data are presented as mean ± SEM (n= 4-6). $^{\#}P< 0.05$, $^{*}P< 0.01$ compared to respective controls.

Time-dependent studies showed that at a concentration of 0.125% (w/v), berry extract down-regulated the gene expression of both GLUT2 and SGLT1 significantly at all time points investigated (4-24 hours) (P= 0.005 or P< 0.001) (**Figure 22**).

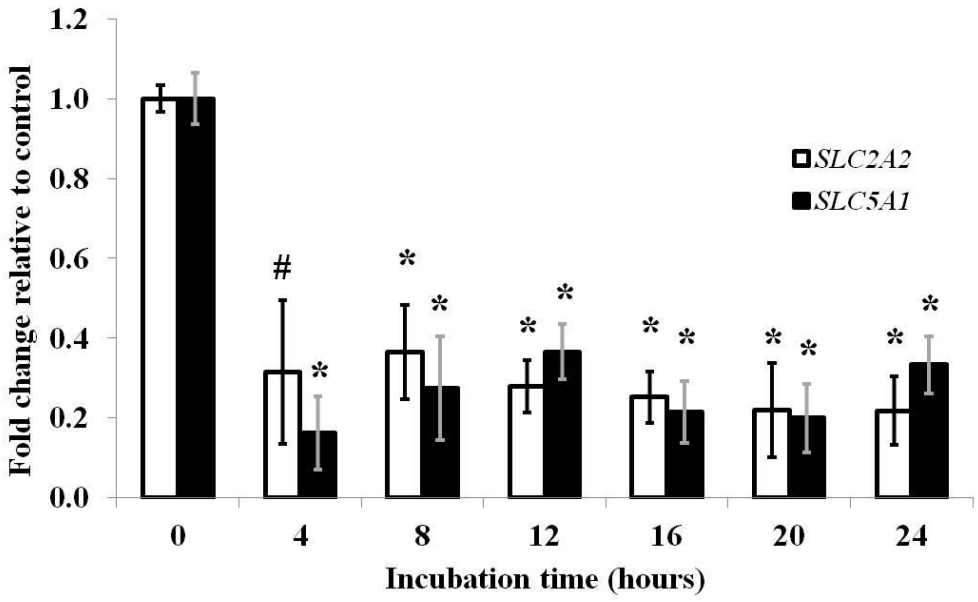

Figure 22. Time dependent effect of berry extract on gene expression of *SLC2A2* (GLUT2) and *SLC5A1* (SGLT1) in Caco-2 cells

Caco-2 cells were treated with 0.125% (w/v) berry extract for different lengths of time. Relative gene expression of *SLC2A2* (GLUT2) and *SLC5A1* (SGLT1) was determined by qRT-PCR. Data are presented as mean ± SEM (n= 12-40). $^{\#}P$= 0.005, *P< 0.001 compared to respective controls.

Quercetin

Dose response effect of the most abundant dietary flavonoid, quercetin, on the gene expression of GLUT2 and SGLT1 in Caco-2 cells was also evaluated. Using qRT-PCR, quercetin was shown to down-regulate the expression of GLUT2 in a dose-dependent manner (6.25-100 μM) (**Figure 23**). The lowest concentration of quercetin used in this study (6.25 μM) reduced the expression of GLUT2 by 28% (P< 0.05) while the highest concentration (100 μM) caused a dramatic 80% decline (P< 0.001) (**Figure 23**). On the other hand, the expression of SGLT1 in response to increasing concentrations of quercetin showed a biphasic change (**Figure 23**). Quercetin at a concentration of 25

μM up-regulated the expression of SGLT1 by 22% ($P< 0.05$) but 100 μM decreased it by 80% ($P< 0.001$) (**Figure 23**).

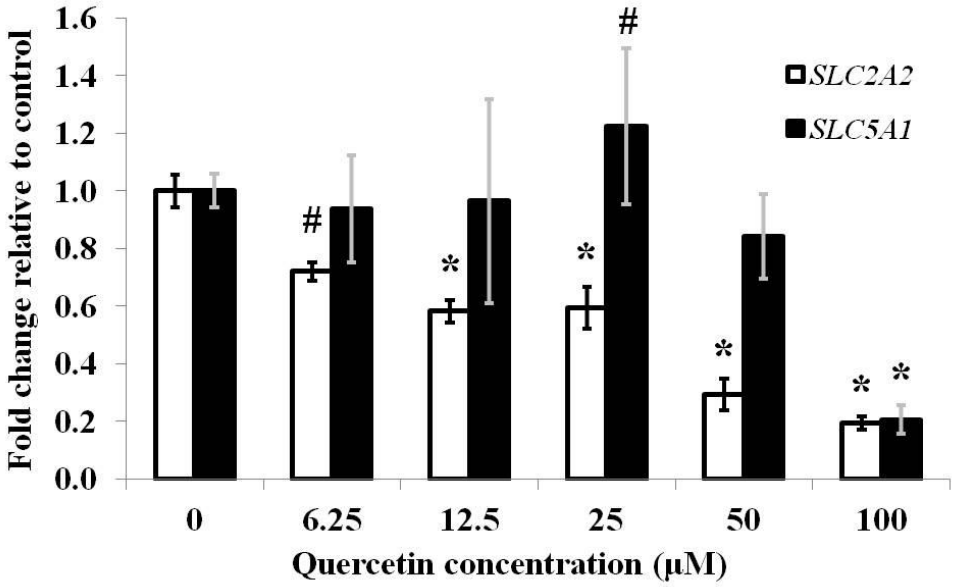

Figure 23. Dose response effect of quercetin on gene expression of *SLC2A2* (GLUT2) and *SLC5A1* (SGLT1) in Caco-2 cells

Caco-2 cells were treated with various concentrations of quercetin (6.25-100 μM) for 16 hours. Relative gene expression of *SLC2A2* (GLUT2) and *SLC5A1* (SGLT1) was determined by qRT-PCR. Data are presented as mean ± SEM (n= 8-34). $^{\#}P< 0.05$, $*P< 0.001$ compared to respective controls.

Investigations into the time course effect of quercetin revealed that the gene expression of GLUT2 in Caco-2 cells was down-regulated significantly after incubation with 100 μM quercetin for 8 hours ($P< 0.05$) and thereafter over the course of the experiment (**Figure 24**). On the other hand, the gene expression of SGLT1 was reduced significantly only at 12 ($P< 0.005$) and 16 hours ($P< 0.001$) of quercetin treatment (**Figure 24**).

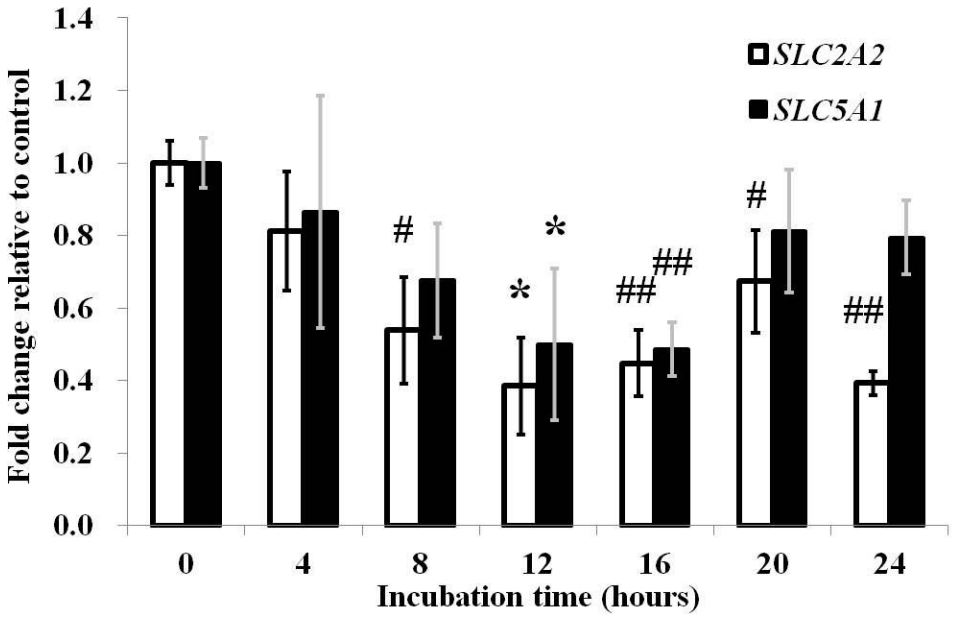

Figure 24. Time dependent effect of quercetin on gene expression of *SLC2A2* (GLUT2) and *SLC5A1* (SGLT1) in Caco-2 cells

Caco-2 cells were treated with 100 μM of quercetin for different lengths of time. Relative gene expression of *SLC2A2* (GLUT2) and *SLC5A1* (SGLT1) was determined by qRT-PCR. Data are presented as mean ± SEM (n= 12-41). $^{\#}P< 0.05$, $^{*}P< 0.005$, $^{\#\#}P< 0.001$ compared to respective controls.

3.1.3.2 Glucose uptake

Berry extract or inhibitors of glucose transporters

There was no significant change in glucose uptake into Caco-2 cells after incubation with 0.125% (w/v) berry extract for 16 hours in both sodium-containing and sodium-free conditions (**Figure 25A** and **B**). Similar results were obtained from glucose uptake studies where Caco-2 cells had been pre-incubated with 100 μM of phloretin, phloridzin or quercetin, i.e. no significant change was observed (**Figure 26A** and **B**).

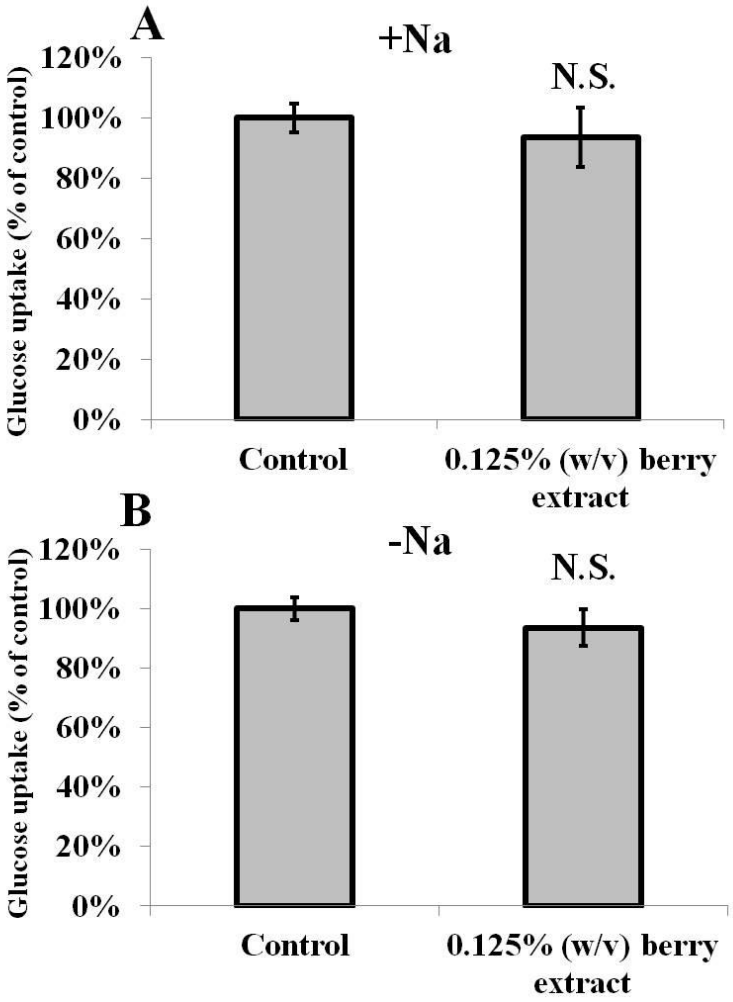

Figure 25. Chronic effect of 0.125% (w/v) berry extract on glucose uptake in Caco-2 cells

Uptake of 1 mM D-glucose with trace of [^{3}H]-labelled D-glucose was measured in the presence (**A**) or absence (**B**) of Na^+ after incubating Caco-2 cells with 0.125% (w/v) berry extract for 16 hours. Data are presented as mean ± SEM (n= 8-9). N.S.- not significant (*P*> 0.05).

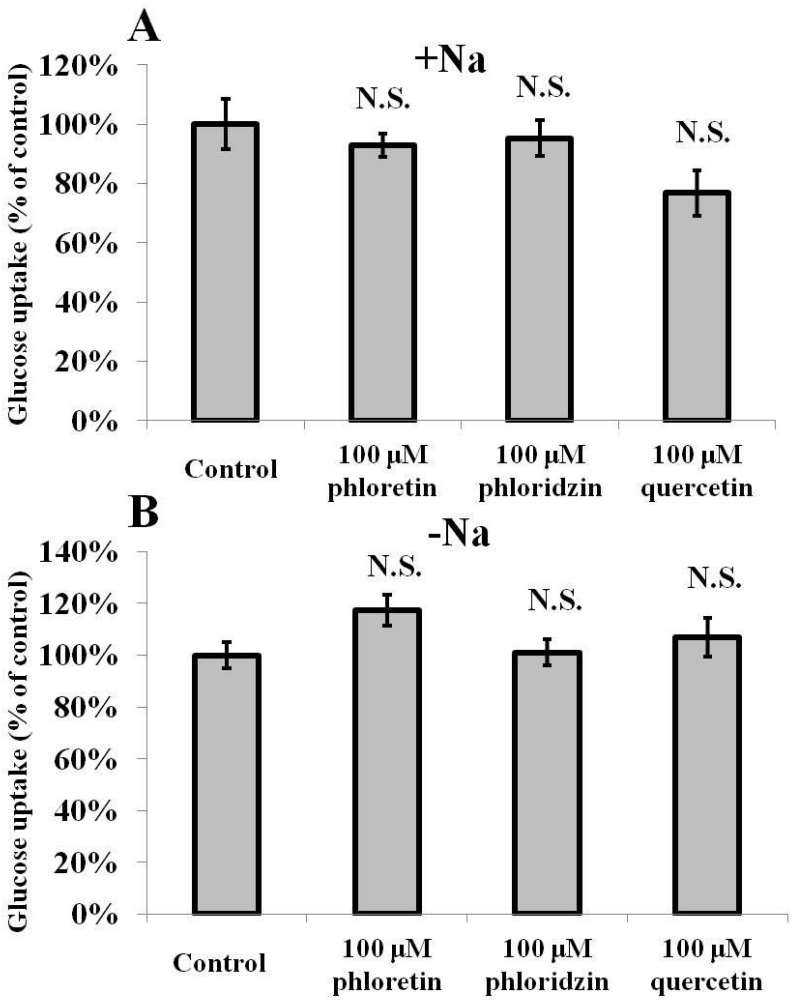

Figure 26. Chronic effect of 100 µM of phloretin, phloridzin or quercetin on glucose uptake in Caco-2 cells

Uptake of 1 mM D-glucose glucose with trace of [^{3}H]-labelled D-glucose was measured in the presence (**A**) or absence (**B**) of Na^+ after incubating Caco-2 cells with 100 µM of phloretin, phloridzin or quercetin for 16 hours. Data are presented as mean ± SEM (n= 6-9). N.S.- not significant (*P*> 0.05).

3.1.3.3 Global miRNA expression

As mentioned in the above section, berry extract at a concentration of 0.125% (w/v) down-regulated the gene expression of GLUT2 and SGLT1. We speculated the involvement of microRNAs (miRNAs), a class of small non-coding RNA molecules, in the transcriptional or post-transcriptional regulation of GLUT2/SGLT1 expression by berry extract.

In this study, using Affymetrix GeneChip® miRNA arrays, changes in global miRNA expression in Caco-2 cells in response to 16-hour treatment with 0.125% (w/v) berry extract were evaluated. The miRNA arrays used in the present study contain probe sets that detect 847 human mature miRNAs. Of these, 347 were present in the human intestinal epithelial Caco-2 cell line (true detection ($P< 0.05$) in at least one group). Upon treatment with 0.125% (w/v) berry extract, 158 miRNAs were differentially expressed by greater than two fold. Specifically, 41 miRNAs were up-regulated (**Table 18**) and 117 were down-regulated (**Table 19**).

Table 18. List of up-regulated miRNAs in Caco-2 cells in response to berry extract treatment

MicroRNA	Fold increase				
miR-1263	23.49	*miR-296-5p*	4.52	*miR-483-3p*	2.55
miR-548a-3p	14.52	*miR-603*	4.37	*miR-891b*	2.53
miR-570	10.17	*miR-518b*	4.00	*miR-220c*	2.52
miR-1323	9.79	*miR-144**	3.95	*let-7f*	2.38
*miR-625**	7.52	*miR-886-3p*	3.82	*miR-1256*	2.37
miR-202	6.58	*miR-220a*	3.81	*miR-1228*	2.36
miR-1272	5.92	*miR-597*	3.27	*miR-920*	2.19
*miR-130b**	5.68	*miR-1234*	3.14	*miR-363**	2.13
miR-606	5.49	*miR-940*	3.12	*let-7a*	2.13
*miR-221**	4.98	*miR-32*	3.09	*miR-583*	2.12
miR-628-5p	4.91	*miR-297*	3.07	*miR-450b-5p*	2.12
*miR-16-2**	4.78	*miR-1294*	3.03	*miR-367*	2.06
miR-451	4.54	*miR-300*	2.93	*miR-1302*	2.05
		miR-1827	2.73	*miR-510*	2.02

Caco-2 cells were treated with 0.125% (w/v) berry extract for 16 hours. Changes in global miRNA expression were determined using miRNA arrays. A two-fold change was set as a threshold.

Following miRNA expression profiling using microarrays, qRT-PCR was performed in an attempt to validate the changes in the miRNA expression shown in the microarray. Five miRNAs were selected, namely *let-7a*, *let-7d*, *let-7f*, *miR-92b* and *miR-106b*. The *let-7* miRNA family has been implicated in the control of glucose metabolism and the pathogenesis of Type II diabetes (Frost and Olson, 2011). Therefore, three miRNAs

from the *let-7* family- *let-7a*, *let-7d* and *let-7f*- which were identified as potential regulators of GLUT2, were chosen. Among these three miRNAs, *let-7a* and *let-7f* were up-regulated as shown in the microarray, while *let-7d* did not change. *miR-106b* was selected as it is also a potential GLUT2 regulator but was down-regulated. For comparison, we also validated the changes in *miR-92b*, which does not seem to bind to either GLUT2 or SGLT1. In other words, *miR-92b* acted as a negative control where no change was expected using qRT-PCR (see **Table 20** for changes in the expression of the selected miRNAs in the microarray).

Table 19. List of down-regulated miRNAs in Caco-2 cells in response to berry extract treatment

MicroRNA	Fold decrease
*miR-138-1**	13.30
miR-346	12.76
miR-134	10.99
miR-503	10.13
miR-345	8.51
miR-1225-5p	8.23
*miR-99b**	8.16
miR-125a-3p	8.07
*miR-425**	7.91
miR-943	7.70
miR-1231	7.57
*miR-93**	7.50
miR-181d	7.15
*miR-424**	7.05
miR-429	6.99
*miR-23a**	6.95
miR-1296	6.91
*miR-92b**	6.85
*miR-378**	6.61
*miR-21**	6.59
miR-885-3p	6.59
*miR-193b**	6.57
miR-720	6.40
miR-454	6.26
miR-330-3p	6.20
miR-491-5p	6.11
miR-615-3p	6.08
miR-339-3p	6.00
*miR-629**	5.98
miR-432	5.70
miR-671-5p	5.67
miR-1290	5.64
miR-1303	5.64
*miR-34a**	5.36
miR-342-3p	5.35
*miR-551b**	5.31
miR-1269	5.23
*miR-150**	4.96
miR-148b	4.92
miR-30e	4.76
miR-455-5p	4.70
miR-374b	4.68
miR-939	4.61
*miR-27a**	4.56
*miR-29b-2**	4.53
miR-212	4.30
miR-1260	4.28
miR-100	4.14
miR-1184	4.14
miR-140-5p	4.13
*miR-373**	4.10
*miR-30e**	3.97
miR-26b	3.78
*miR-18a**	3.78
miR-324-3p	3.78
miR-1202	3.73
miR-188-5p	3.71
miR-184	3.70
miR-195	3.66
miR-933	3.49
miR-1280	3.39
miR-138	3.35
miR-618	3.34
*miR-10b**	3.30
miR-769-5p	3.28
miR-1226	3.27
miR-339-5p	3.23
miR-1262	3.18
miR-30a	3.14
miR-149	3.12
miR-1274b	3.07
miR-301a	3.04
miR-484	2.93
*miR-24-2**	2.92
miR-1825	2.87
miR-941	2.87
miR-665	2.85
miR-638	2.85
miR-1285	2.82
miR-338-3p	2.76
miR-133a	2.75
*miR-30b**	2.71
miR-139-3p	2.69
*miR-22**	2.67
miR-22	2.65
*miR-10a**	2.59
*miR-92a-1**	2.55
*miR-200a**	2.55
miR-1250	2.54
*miR-589**	2.53
miR-198	2.52
miR-500	2.50
miR-203	2.37
miR-551a	2.37
miR-452	2.34
miR-1292	2.32
*miR-30a**	2.30
miR-1207-5p	2.30
*miR-185**	2.28
miR-641	2.26
*miR-664**	2.22
miR-1307	2.21
miR-152	2.18
miR-502-5p	2.17
miR-885-5p	2.16
miR-768-5p	2.14
miR-550	2.14
miR-1268	2.13
miR-501-5p	2.11
*miR-1228**	2.11
*miR-135b**	2.09
miR-140-3p	2.08
miR-106b	2.07
miR-7	2.06
miR-553	2.06
*miR-149**	2.06
*miR-200c**	2.06

Caco-2 cells were treated with 0.125% (w/v) berry extract for 16 hours. Changes in global miRNA expression were determined using miRNA arrays. A two-fold change was set as a threshold.

Validation of miRNA microarray data by qRT-PCR

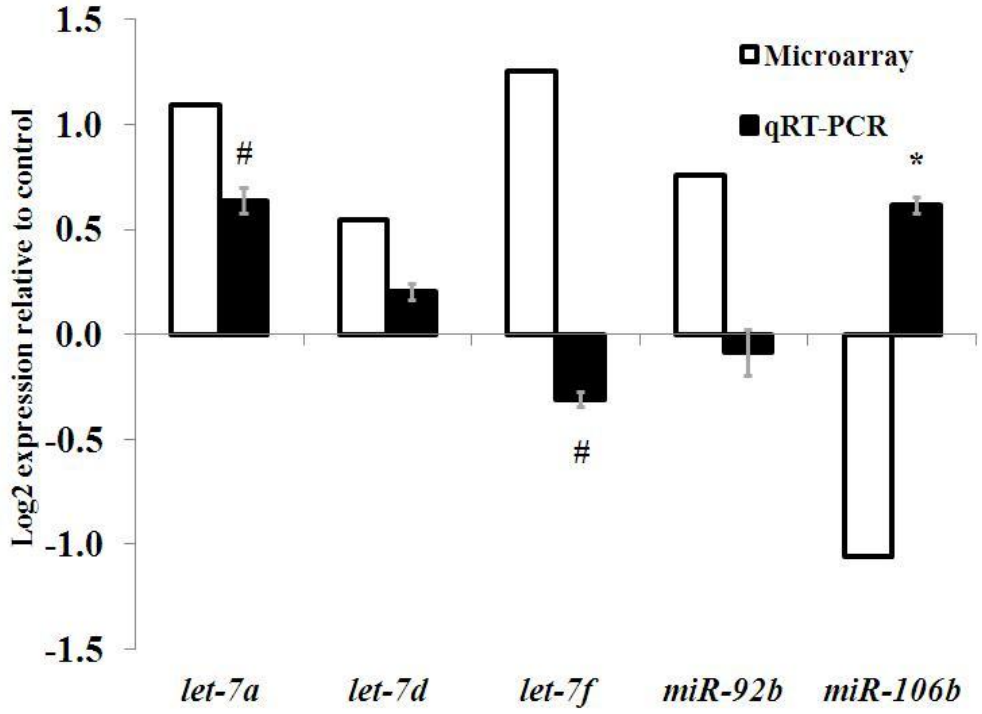

Figure 27. Validation of miRNA microarray data for selected miRNAs by qRT-PCR

Five miRNAs- *let-7a*, *let-7d*, *let-7f*, *miR-92b* and *miR-106b*- were selected for validation by qRT-PCR. Microarray: data are presented as mean; qRT-PCR: data are presented as mean ± SEM (n= 8). $^{\#}P< 0.005$, $^{*}P< 0.001$ compared with control.

Table 20. Changes in the expression of the five selected miRNAs in the microarray and in qRT-PCR

MicroRNA	Change call in microarray	Change in qRT-PCR (*P*-value)
let-7a	Increase	Increase ($P= 0.004$)
let-7d	No change	No change ($P= 0.164$)
let-7f	Increase	Decrease ($P= 0.001$)
miR-92b	No change	No change ($P= 0.618$)
miR-106b	Decrease	Increase ($P< 0.001$)

Results from qRT-PCR did not validate the data obtained from miRNA microarray (see **Table 20**), i.e. direction of changes of two out of five selected miRNAs, namely *let-7f* and *miR-106b*, are opposite in the microarray and in the qRT-PCR. Nevertheless, we found an up-regulation in the expression of *let-7a* in both microarray and qRT-PCR. Using the bioinformatics algorithm, *TargetScanHuman version 6.2*, the mRNA of

GLUT2 is predicted to be one of the targets of *let-7a*. **Figure 28** shows the predicted consequential pairing of the 3'-UTR of GLUT2 mRNA and *let-7a* miRNA.

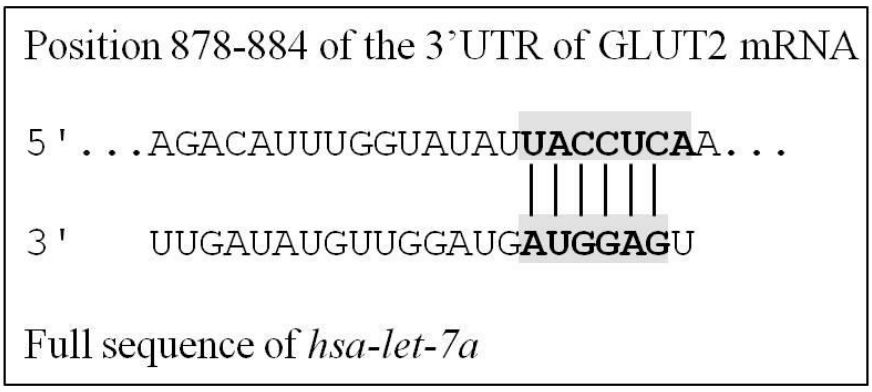

Figure 28. Interaction between the 3'UTR of GLUT2 mRNA and *let-7a* miRNA

The match type between *SLC2A2* and *let-7a* miRNA belongs to the 7mer-A1 type, i.e. base-pairing between the seed regions (nucleotides 2-7 from 5' end) of the miRNA and the conserved sites in the 3'UTR (3'-untranslated region) of mRNAs and an A across from nucleotide 1 (highlighted in gray). Prediction of miRNA target was performed using the bioinformatics algorithm, *TargetScanHuman version 6.2* (http://www.targetscan.org/).

3.2 C2C12 muscle cells

3.2.1 Characterisation of the C2C12 muscle cells

3.2.1.1 Giemsa staining of the C2C12 muscle cells

Differentiation of C2C12 myoblasts into myotubes was monitored by Giemsa staining. Giemsa stain (BDH, UK) stains the nuclei pink, the chromosomes dark purple, the cytoplasm pale gray-pink and the protein-rich myotubes dark red-purple.

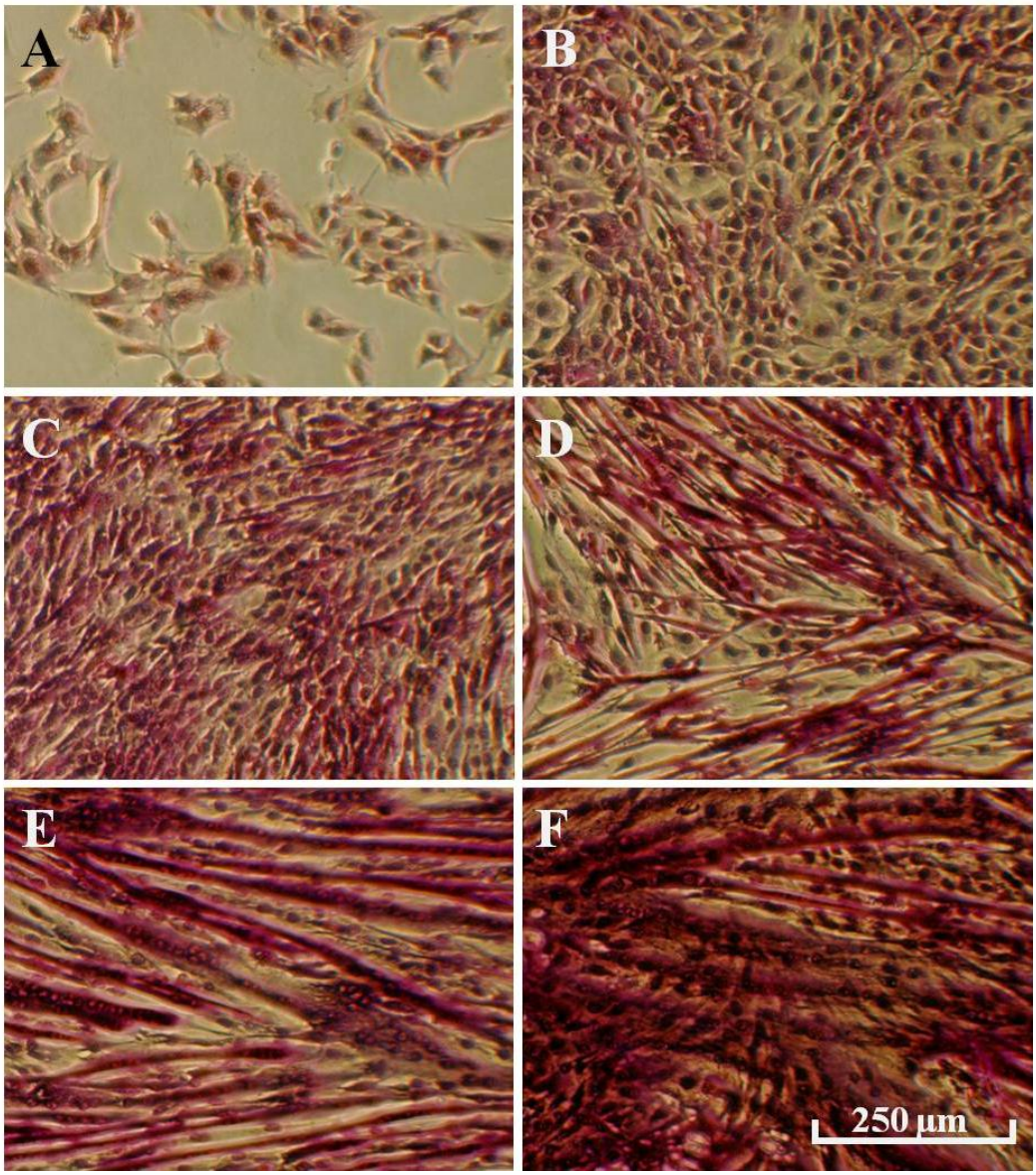

Figure 29. Giemsa staining of C2C12 cells during differentiation (100X)

C2C12 cells were differentiated at Day 0. Images shown were C2C12 cells at Day -1 (**A**), Day 0 (**B**), Day 1 (**C**), Day 3 (**D**), Day 5 (**E**) and Day 7 (**F**) (100X magnification).

At Day -1 (1-2 days post-seeding and 1 day before initiation of differentiation), C2C12 myoblasts appeared as fusiform or star-shaped mononucleated cells (**Figure 29A** and **Figure 30A**). The myoblasts proliferated and reached about 90-100% confluence at Day 0 (**Figure 29B** and **Figure 30B**). At Day 0, myogenic differentiation was induced by switching fetal bovine serum-supplemented cell culture medium to horse serum. One day after the change of serum supplementation (Day 1), the C2C12 cells were characterised by lengthening and appearance of thin dark-red-purple-stained filaments, indicating formation of protein-rich muscle fibres (**Figure 29C** and **Figure 30C**). By Day 3, mononucleated cells began to fuse and become multinucleated, forming long myotubes (**Figure 29D** and **Figure 30D**). From Day 5 onwards, myogenesis increased progressively as the myotubes thickened, branched and frequently overpassed the underlying mononucleated myoblasts (**Figure 29E** and **F** and **Figure 30E** and **F**).

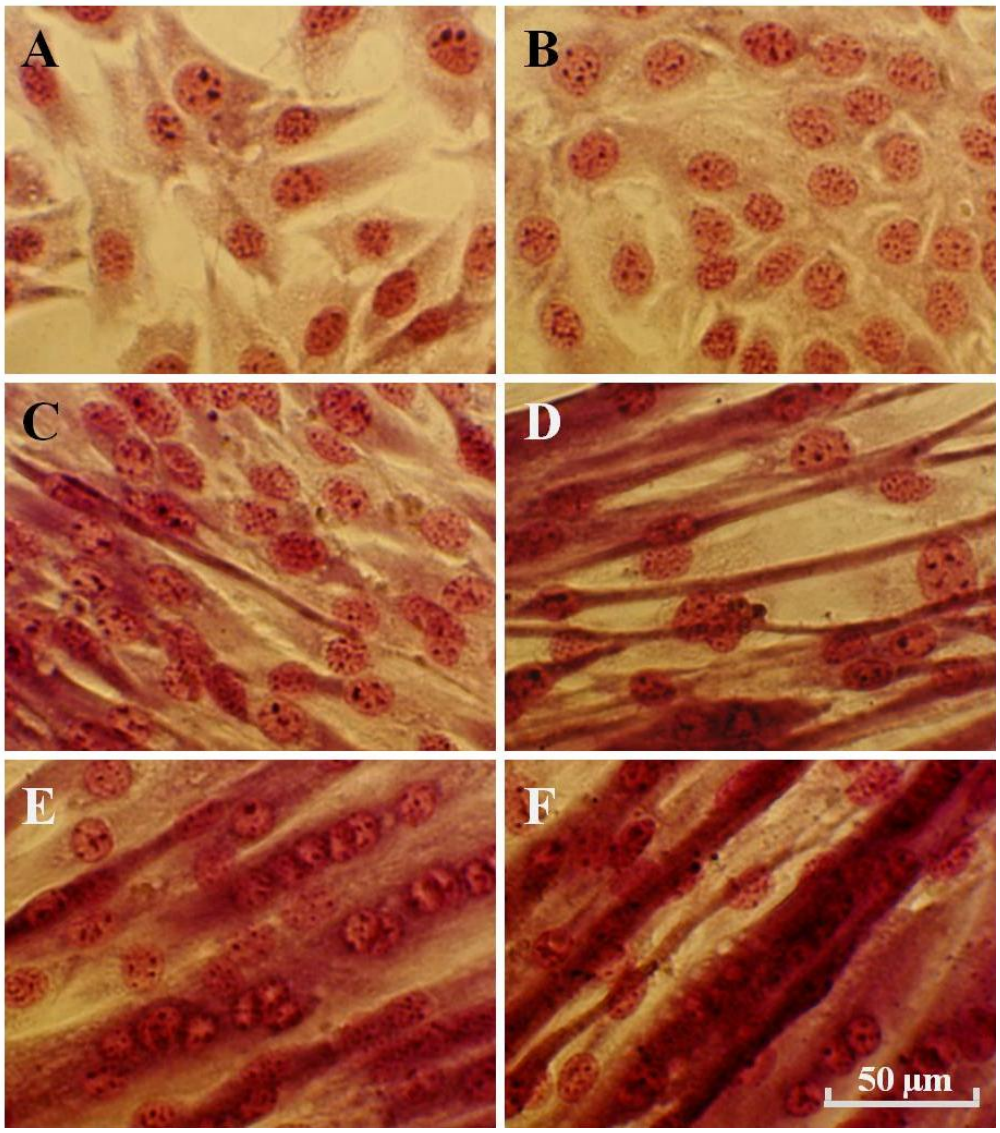

Figure 30. Giemsa staining of C2C12 cells during differentiation (400X)

C2C12 cells were differentiated at Day 0. Images shown were C2C12 cells at Day -1 (**A**), Day 0 (**B**), Day 1 (**C**), Day 3 (**D**), Day 5 (**E**) and Day 7 (**F**) (400X magnification).

3.2.1.2 Gene expression of glucose transporters

Changes in the gene expression of the two main glucose transporters in muscle cells, GLUT1 and GLUT4, were evaluated over the course of culture during C2C12 differentiation. At Day-1, a time point where C2C12 cells appeared as actively proliferating myoblasts, gene expression of GLUT1 was relatively high compared to that of GLUT4 (**Figure 31**). Upon reaching confluence at Day 0, although the cells still existed as undifferentiated myoblasts, a dramatic decline in the expression of GLUT1

($P< 0.001$) and a sudden rise in the expression of GLUT4 ($P< 0.001$) were already observed (**Figure 31**). There were no further changes in the expression of both GLUT1 and GLUT4 from Day 0 to Day 7.

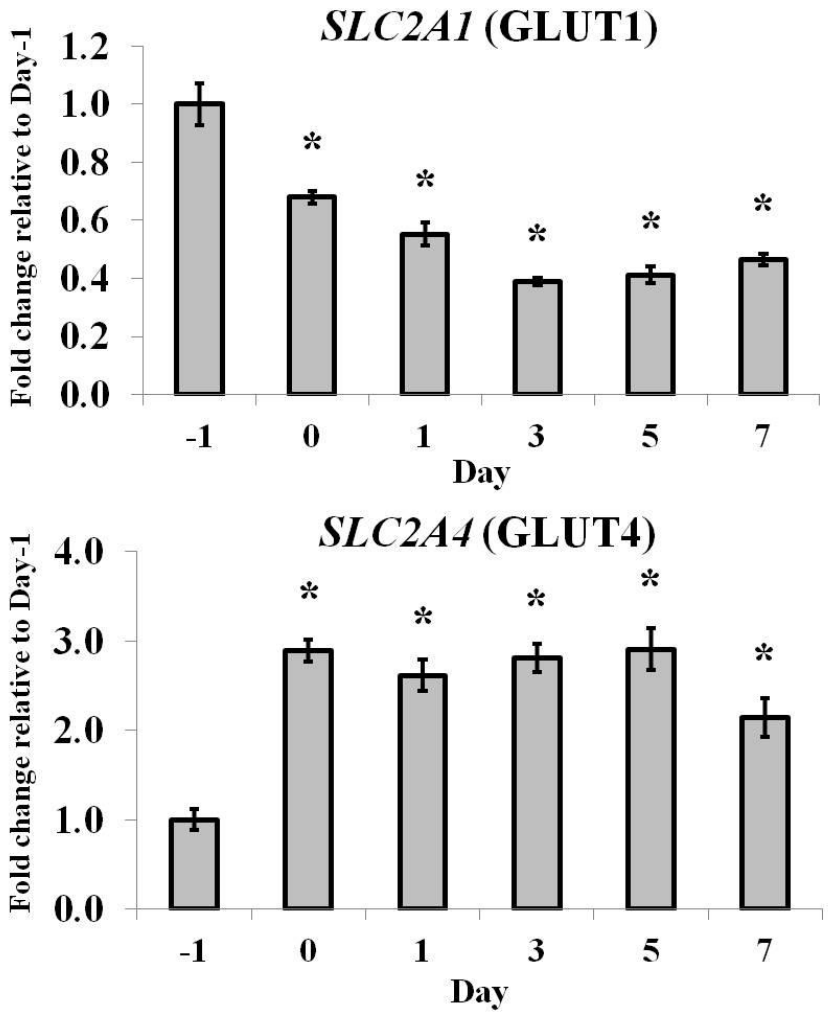

Figure 31. Relative gene expression of *SLC2A1* and *4* of during C2C12 differentiation

Relative gene expression of *SLC2A1* and *4* (GLUT1 and 4) was determined by qRT-PCR. Data are presented as mean ± SEM (n= 4). $^{*}P< 0.001$ compared with expression at Day -1. C2C12 cells reached 90-100% confluence and were initiated to undergo differentiation at Day 0.

Table 21. Ct values of *SLC2A1* and *4* in C2C12 cells at Day 7 obtained from qRT-PCR

Gene name (Encoded protein)	Ct value at Day 7 (Mean ± SEM, n=4)
SLC2A1 (GLUT1)	21.33 ± 0.04
SLC2A4 (GLUT4)	27.25 ± 0.08

3.2.2 Cell viability test

3.2.2.1 Trypan blue exclusion

Trypan blue is a diazo dye that distinguishes between viable and non-viable cells by staining non-viable cells that do not possess intact membrane blue (Strober, 2001). Using this trypan blue dye exclusion method, C2C12 cells were shown to be >80% viable after treatments with 3-300 mM ethanol for 24 hours (**Figure 32**).

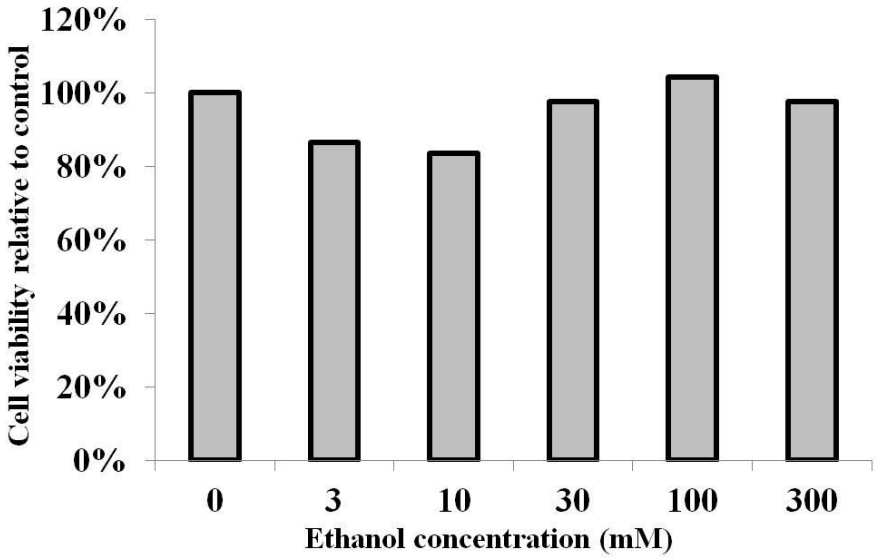

Figure 32. Trypan blue test for cell viability of C2C12 myotubes upon treatment with various concentrations of ethanol (0-300 mM)

Data are obtained based on counting a total of 200-400 cells from a representative sample chosen at random for each group.

3.2.2.2 MTS viability assay

MTS (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium) is a yellow tetrazolium compound that, in metabolically active cells, is reduced to a purple-coloured formazan product that give a maximum absorbance at 490 nm. Results from trypan blue test for ethanol treatment on cell viability of C2C12 cells were confirmed using the MTS viability assay. No significant changes in cell viability were observed after treating C2C12 cells with 3-300 mM ethanol for 24 hours ($P > 0.05$) (**Figure 33**).

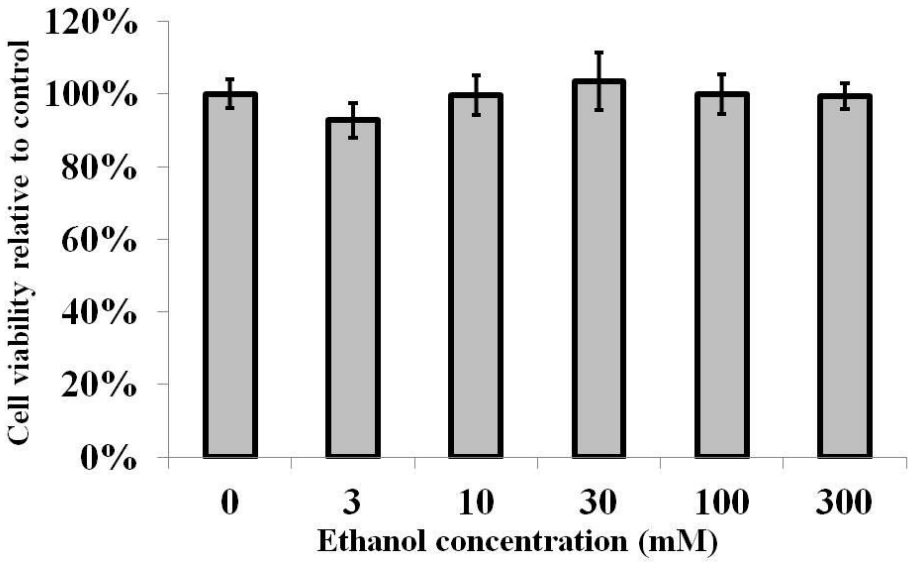

Figure 33. MTS assay for cell viability of C2C12 myotubes upon treatment with various concentrations of ethanol (0-300 mM)

Data are presented as mean ± SEM (n= 7-8).

Similarly, the MTS assay showed that there were no significant changes in C2C12 cell viability after incubation with 0.008-0.125% (w/v) berry extract for 24 hours ($P > 0.05$) (**Figure 34**).

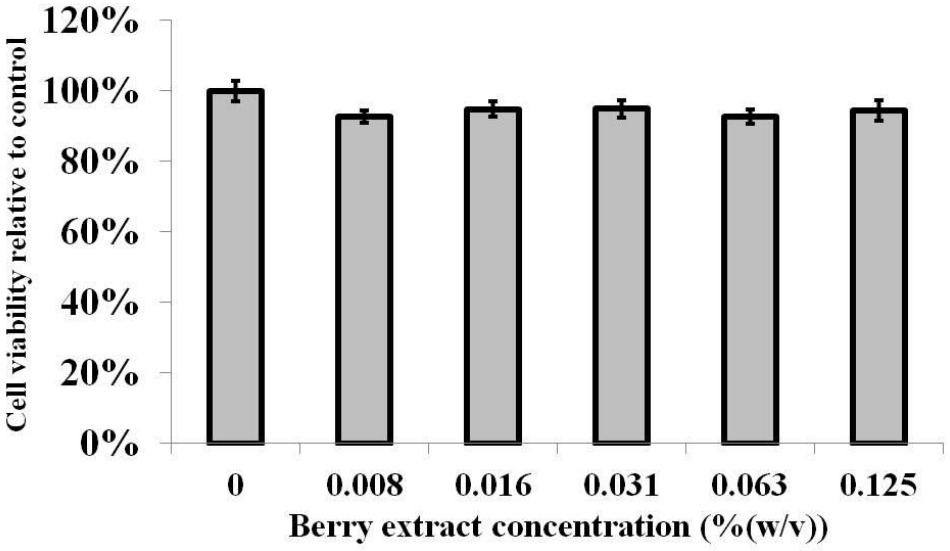

Figure 34. MTS assay for cell viability of C2C12 myotubes upon treatment with various concentrations of berry extract (0-0.125% (w/v))

Data are presented as mean ± SEM (n= 4).

3.2.3 Effect of berry extract on glucose uptake in the C2C12 myotubes

3.2.3.1 Gene expression of glucose transporters

At the lowest concentration of berry extract (0.008% (w/v)), gene expression of GLUT1 was decreased by 20% ($P< 0.05$) (**Figure 35**). In contrast, 0.125% (w/v) berry extract, the highest concentration investigated, increased it by 30% ($P< 0.05$). On the other hand, berry extract did not cause any significant changes in the gene expression of GLUT4 ($P> 0.05$) (**Figure 35**).

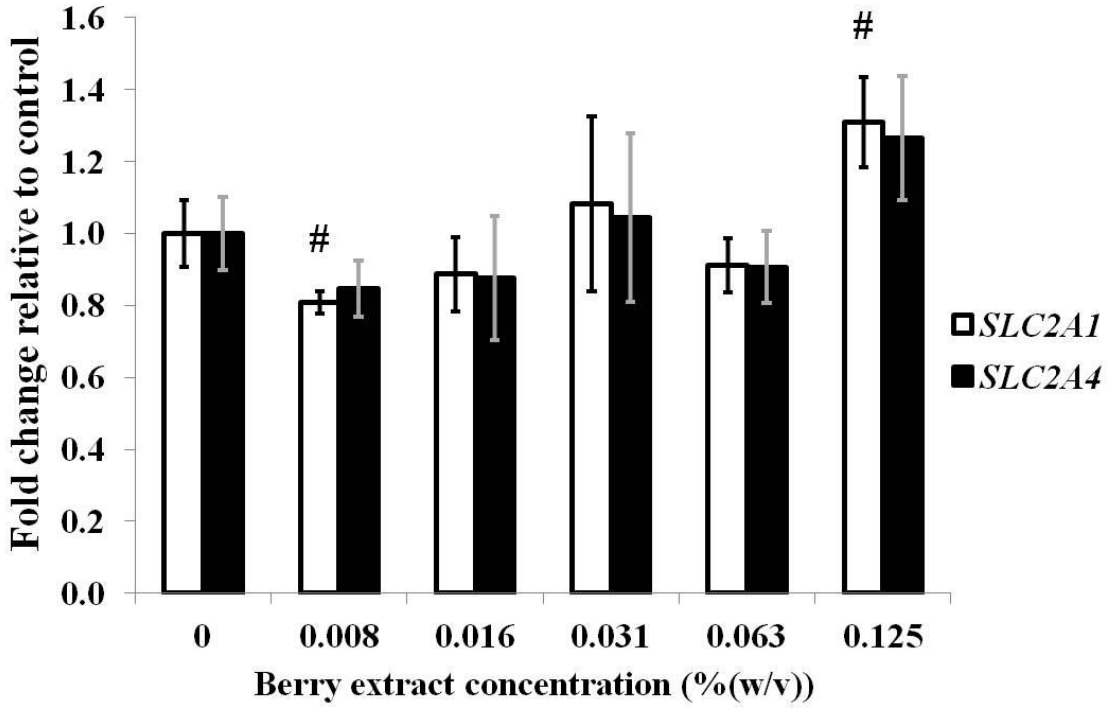

Figure 35. Dose response effect of berry extract on gene expression of *SLC2A1* and *4* (GLUT1 and 4) in C2C12 myotubes

C2C12 myotubes were treated with various concentrations of berry extract (0-0.125% (w/v)) for 24 hours. Relative gene expression of *SLC2A1* and *4* (GLUT1 and 4) was determined by qRT-PCR. Data are presented as mean ± SEM (n= 11-12). $^{\#}P< 0.05$ compared to control.

3.2.3.2 Morphology of C2C12 myotubes pre-treated with insulin

Morphology of C2C12 myotubes with or without 3-day insulin treatment was compared. In C2C12 cells subjected to incubation with 100 nM insulin for 3 days (**Figure 36B**), a more extensive development of lengthened multinucleated myotube

network was observed. Thus, the amount of myotubes was apparently higher, and the myotubes were more branched but thinner (smaller diameter) compared to the untreated cells (**Figure 36A**). In addition, based on personal observations not shown here, spontaneous twitching was more vigorous in the insulin-treated myotubes and the metabolic rate also appeared to be higher, which was determined by a more orange colour of the cell culture medium (a drop in pH) indicating higher rate of acidic metabolic waste production.

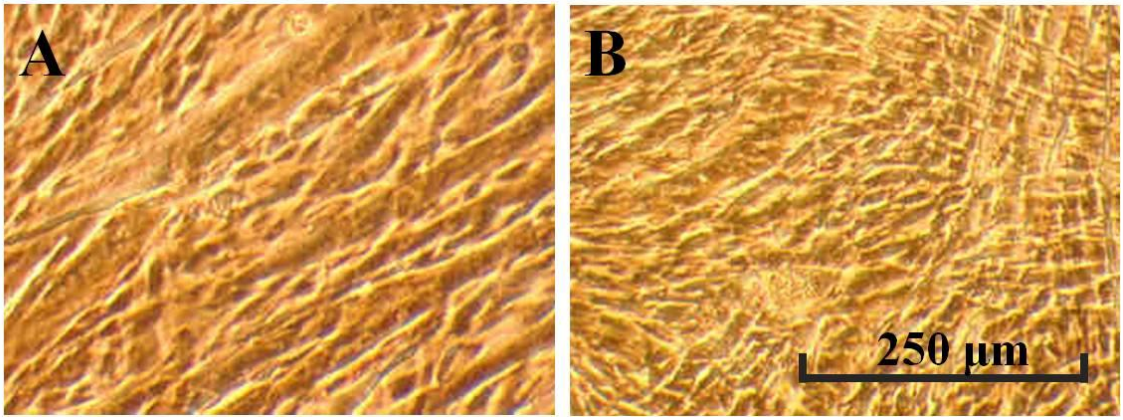

Figure 36. C2C12 myotubes at Day 7 with or without insulin pre-treatment
C2C12 cells were differentiated at Day 0. Images shown were C2C12 myotubes at Day 7 without pre-treatment (**A**) or pre-treated with 100 nM insulin for 3 days (**B**) (100X magnification).

3.2.3.3 Gene expression of glucose transporters after 3-day insulin pre-treatment

In C2C12 myotubes without insulin pre-treatment (white bars in **Figure 37**), 0.125% (w/v) berry extract did not induce significant changes in the gene expression of GLUT1 and GLUT4 ($P > 0.05$). Comparing the glucose transporter expression in C2C12 myotubes with or without 3-day insulin pre-treatment (black bars versus white bars in the control groups in **Figure 37**), the expression of GLUT4 was massively up-regulated by ~2.7-fold by insulin pre-treatment ($P < 0.001$). The expression of GLUT1, in

contrast, decreased by more than 20%, albeit not reaching statistical significance in one-way ANOVA ($P > 0.05$, Tukey's post-hoc test).

In C2C12 myotubes which had been pre-treated with insulin (black bars in **Figure 37**), 0.125% (w/v) berry extract caused a ~2.4-fold up-regulation of the gene expression of GLUT1 ($P < 0.001$). However, expression of GLUT4 did not show significant changes under the same condition ($P > 0.05$).

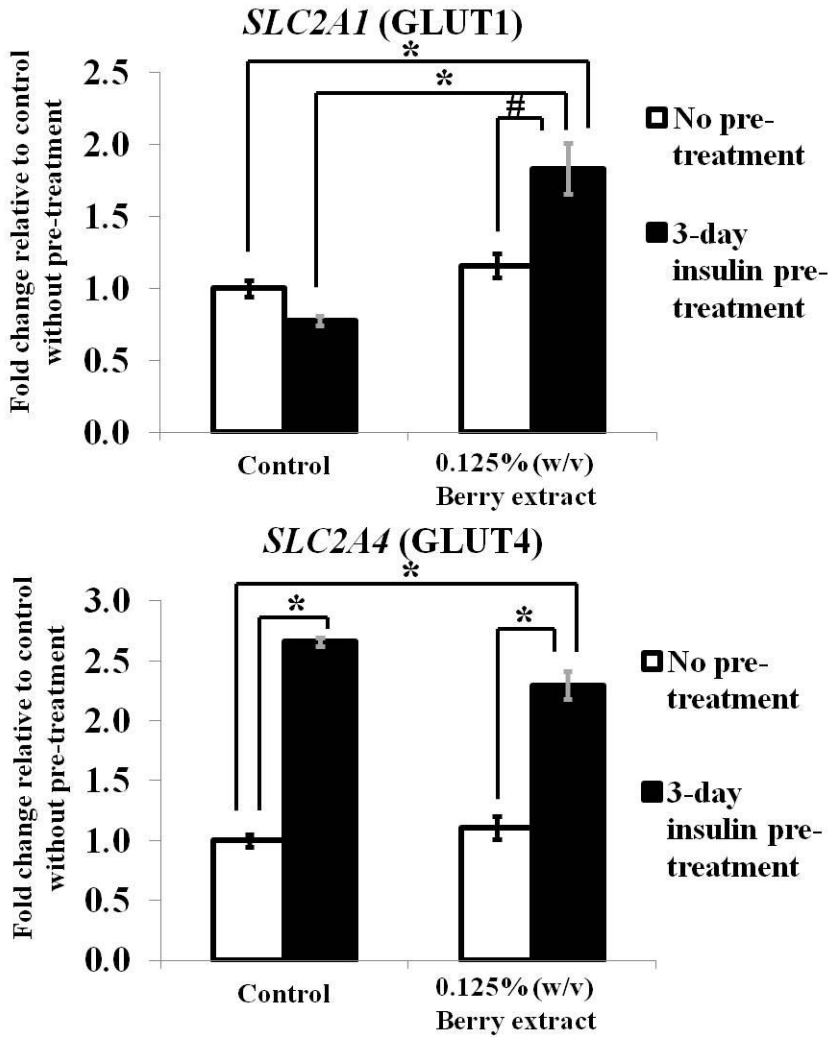

Figure 37. Relative gene expression of *SLC2A1* and *4* (GLUT1 and 4) after 24-hour treatment with 0.125% (w/v) berry extract in C2C12 myotubes

C2C12 myotubes without pre-treatment or pre-treated with 100 nM insulin for 3 days were subjected to 24-hour incubation with 0.125% (w/v) berry extract. Relative gene expression of *SLC2A1* and *4* (GLUT1 and 4) was determined by qRT-PCR. Data presented are mean ± SEM (n= 4-6). $^{\#}P = 0.001$, $^{*}P < 0.001$.

3.2.3.4 Glucose uptake under basal or insulin-stimulated conditions

In C2C12 myotubes that had not been subjected to insulin pre-incubation (**Figure 38A**), 0.125% (w/v) berry extract did not cause significant changes in glucose uptake, both in basal and insulin-stimulated conditions ($P > 0.05$). It is noteworthy that stimulation with 100 nM insulin alone for 30 minutes also did not significantly alter glucose uptake in C2C12 myotubes ($P > 0.05$) (**Figure 38A**).

In C2C12 myotubes that had been pre-treated with 100 nM insulin for 3 days (**Figure 38B**), the cells became insulin-responsive, i.e. insulin itself was able to stimulate glucose uptake (a 25% increase ($P < 0.001$)) (black bar versus white bar in control group in **Figure 38B**). Berry extract at a concentration of 0.125% (w/v), on the other hand, did not alter basal glucose uptake in insulin-pre-treated C2C12 myotubes ($P > 0.05$). There were also no significant changes in insulin-induced glucose uptake in cells treated with 0.125% (w/v) berry extract, compared to 30-minute insulin incubation alone ($P > 0.05$) (black bars in **Figure 38B**).

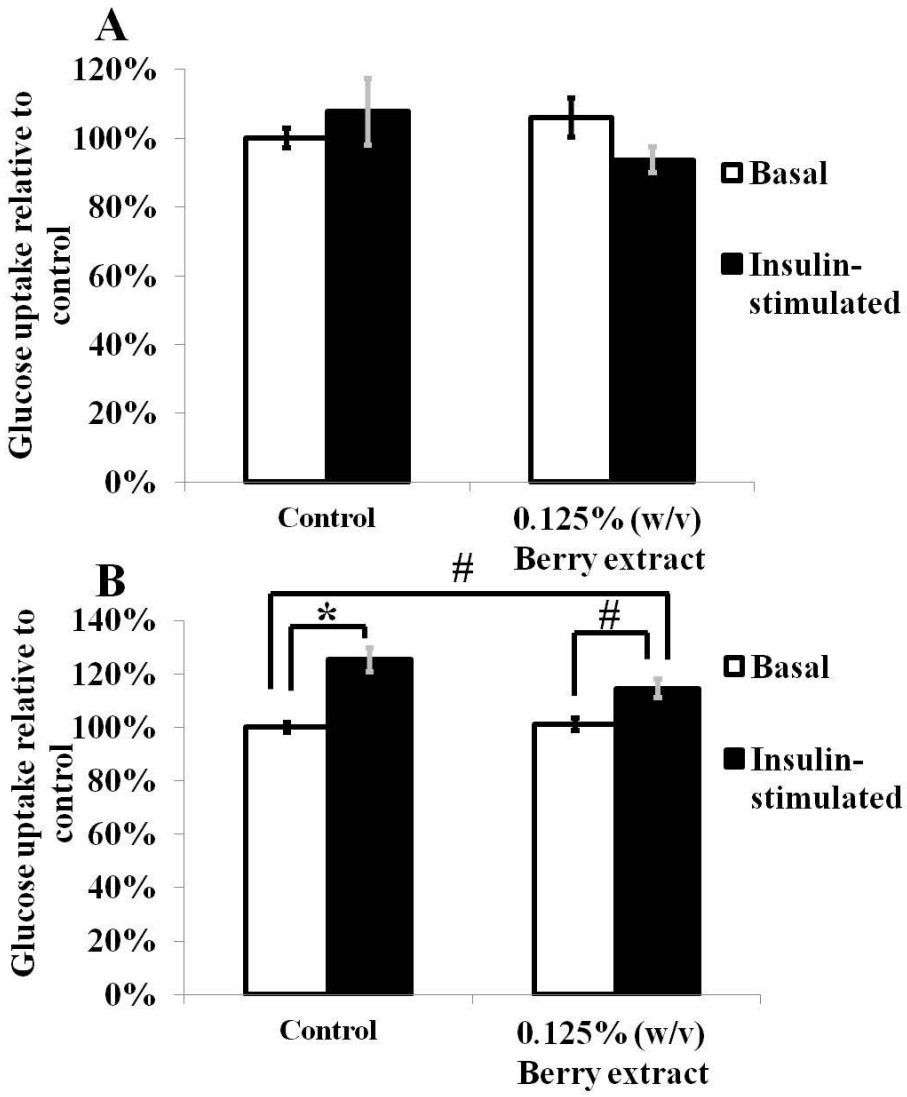

Figure 38. Effects of 0.125% (w/v) berry extract (24 hours) on glucose uptake under basal or insulin-stimulated conditions in C2C12 myotubes

C2C12 myotubes without pre-treatment (**A**) or pre-treated with insulin for 3 days (**B**) were treated with or without 0.125% (w/v) berry extract for 24 hours. Uptake of 1 mM D-glucose with trace of [^{3}H]-labelled D-glucose was measured under basal or insulin-stimulated conditions. Under insulin-stimulated condition, cells were pre-incubated with 100 nM insulin for 30 minutes and uptake was measured in the presence of insulin. Data are presented as mean ± SEM (n= 6). $^{\#}P< 0.05$, $^{*}P< 0.001$.

3.2.4 Effect of ethanol on glucose transporter expression in the C2C12 myotubes

Ethanol at concentrations ranging from 3 mM to 300 mM did not trigger any changes in the gene expression of GLUT1 in C2C12 myotubes ($P> 0.05$) (**Figure 39**). On the

other hand, at high concentrations (30, 100 and 300 mM), ethanol significantly suppressed the mRNA level of GLUT4, all by ~20% ($P< 0.05$) (**Figure 39**).

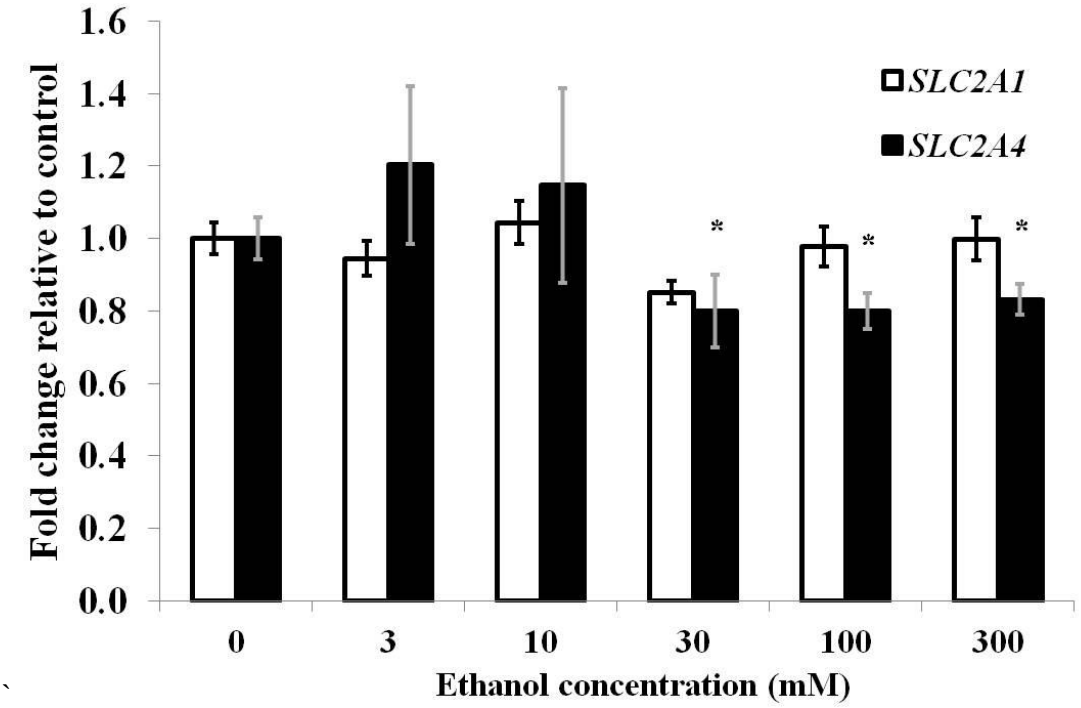

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Figure 39. Dose response effect of ethanol on gene expression of *SLC2A1* and *4* (GLUT1 and 4) in C2C12 myotubes

C2C12 myotubes were treated with various concentrations of ethanol (0-300 mM) for 24 hours. Relative gene expression of *SLC2A1* and *4* (GLUT1 and 4) was determined by qRT-PCR. Data are presented as mean ± SEM (n= 11-12). *$P< 0.05$ compared to respective controls.

3.2.5 Global mRNA expression of the C2C12 myotubes

3.2.5.1 Berry extract

The global gene expression profile of C2C12 myotubes following exposure to 0.125% (w/v) berry extract for 24 hours was investigated using the Affymetrix Mouse Genome 430A microarrays. A two-fold change relative to control (signal log ratio ≥ 1.0 or ≤ -1.0) was selected as a threshold to define differentially expressed genes. Upon treatment with berry extract, the expression of 488 genes was altered by greater than 2-fold, among which 136 were up-regulated and 352 were down-regulated. Using the DAVID v6.7 gene functional classification tool (medium classification stringency) as

well as independent literature search, functionally-related altered genes were categorised into 7 groups, namely glutathione metabolism, transcriptional regulation, nucleic acid binding/ processing, membrane transport, signal peptide, leucine-rich repeat and extracellular structure/ cytoskeleton. Data are presented in **Table 22**. A full list of all 488 differentially expressed genes is presented in **Table 26**, page 203 and **Table 27**, page 207 in the Appendix.

3.2.5.2 Ethanol

The global gene expression profile of C2C12 myotubes following exposure to 300 mM ethanol for 24 hours was investigated using the Affymetrix Mouse Genome 430A microarrays. Using a threshold cut-off at 2-fold change (signal log ratio ≥ 1.0 or ≤ -1.0), we only identified 36 differentially expressed genes, while the detection signals for about half of these genes were very close to background (signal intensities< 50). Therefore, a 1.4-fold change relative to control (signal log ratio ≥ 0.5 or ≤ -0.5) was tentatively selected as a threshold to define differentially expressed genes. Upon treatment with ethanol, the expression of 239 genes was altered by greater than 1.4-fold, among which 96 were up-regulated and 143 were down-regulated. Using the DAVID v6.7 gene functional classification tool (medium classification stringency) as well as independent literature search, functionally-related altered genes were categorised into 5 groups, namely insulin signalling/ glucose metabolism, muscle metabolism and contraction, transcription factor, cell cycle and protein synthesis. Data are presented in **Table 23**. A full list of all 239 differentially expressed genes is presented in **Table 28**, page 217 and **Table 29**, page 220 in the Appendix.

Table 22. List of functionally-related genes differentially expressed upon treatment with berry extract in C2C12 myotubes

Affymetrix ID	Gene symbol	Functional category/ gene description	Signal log ratio	Fold change
		Glutathione metabolism		
1448354_at	*G6PDX*	glucose-6-phosphate dehydrogenase X-linked	1.1	2.14
1418627_at	*GCLM*	glutamate-cysteine ligase, modifier subunit	1.6	3.03
1421041_s_at	*GSTA1*	glutathione S-transferase, alpha 1 (Ya)	2.5	5.66
1421040_a_at	*GSTA2*	glutathione S-transferase, alpha 2 (Yc2)	3.7	13.00
1423436_at	*GSTA3*	glutathione S-transferase, alpha 3	4.7	25.99
1416368_at	*GSTA4*	glutathione S-transferase, alpha 4	1.8	3.48
1416416_x_at	*GSTM1*	glutathione S-transferase, mu 1	1.4	2.64
1415897_a_at	*MGST1*	microsomal glutathione S-transferase 1	1.2	2.30
1452592_at	*MGST2*	microsomal glutathione S-transferase 2	1.2	2.30
		Transcriptional regulation		
1449363_at	*ATF3*	activating transcription factor 3	1.2	2.30
1418982_at	*CEBPA*	CCAAT/ enhancer binding protein (C/EBP), alpha	1.0	2.00
1427844_a_at	*CEBPB*	CCAAT/ enhancer binding protein (C/EBP), beta	1.4	2.64
1423233_at	*CEBPD*	CCAAT/ enhancer binding protein (C/EBP), delta	1.8	3.48
1437247_at	*FOSL2*	fos-like antigen 2	1.1	2.14
1418936_at	*MAFF*	v-maf musculoaponeurotic fibrosarcoma oncogene family, protein F	1.1	2.14
1426538_a_at	*TP53*	tumour suppressor protein p53	1.0	2.00
1433863_at	*BTF3*	basic transcription factor 3	-1.0	0.50
1419959_s_at	*CPHX*	cytoplasmic polyadenylated homeobox	-1.5	0.35
1425698_a_at	*CREBZF*	CREB/ATF bZIP transcription factor	-1.3	0.41
1436434_at	*E2F2*	E2F transcription factor 2	-1.4	0.38
1437820_at	*FOXS1*	forkhead box S1	-1.1	0.47
1433640_at	*FUBP1*	far upstream element (FUSE) binding protein 1	-1.0	0.50
1451285_at	*FUS*	fusion, derived from t(12;16) malignant liposarcoma (human)	-1.1	0.47
1418007_at	*GCFC1*	GC-rich sequence DNA-binding factor 1	-1.0	0.50

To be cont'd

Affymetrix ID	Gene symbol	Functional category/ gene description	Signal log ratio	Fold change
1436932_at	*GRHL3*	grainyhead-like 3 (Drosophila)	-2.6	0.16
1419302_at	*HEYL*	hairy/enhancer-of-split related with YRPW motif-like	-1.1	0.47
1418152_at	*HMGN5*	high-mobility group nucleosome binding domain 5	-2.4	0.19
1424252_at	*HNRPDL*	heterogeneous nuclear ribonucleoprotein D-like	-1.5	0.35
1451776_s_at	*HOPX*	HOP homeobox	-1.3	0.41
1439885_at	*HOXC5*	homeo box C5	-1.3	0.41
1416630_at	*ID3*	inhibitor of DNA binding 3	-1.7	0.31
1418301_at	*IRF6*	interferon regulatory factor 6	-1.2	0.44
1439847_s_at	*KLF12*	Kruppel-like factor 12	-1.6	0.33
1421028_a_at	*MEF2C*	myocyte enhancer factor 2C	-1.0	0.50
1452349_x_at	*MNDA*	myeloid cell nuclear differentiation antigen	-1.0	0.50
1434830_at	*MXD1*	MAX dimerization protein 1	-1.1	0.47
1444980_at	*ONECUT2*	one cut domain, family member 2	-1.2	0.44
1442148_at	*PSIP1*	PC4 and SFRS1 interacting protein 1	-1.1	0.47
1432232_at	*RCOR3*	REST corepressor 3	-1.0	0.50
1427988_s_at	*SAFB2*	scaffold attachment factor B2	-1.0	0.50
1427417_at	*SCML4*	sex comb on midleg-like 4 (Drosophila)	-2.0	0.25
1416778_at	*SDPR*	serum deprivation response	-1.0	0.50
1430271_x_at	*TAF1D*	TATA box binding protein (Tbp)-associated factor, RNA polymerase I, D	-1.1	0.47
1428467_at	*TARDBP*	TAR DNA binding protein	-1.1	0.47
1450961_a_at	*TCEAL5*	transcription elongation factor A (SII)-like 5	-2.3	0.20
1435585_at	*TCEAL7*	transcription elongation factor A (SII)-like 7	-1.1	0.47
		Nucleic acid binding/ processing		
1455936_a_at	*RBPMS*	RNA binding protein gene with multiple splicing	1.4	2.64
1455740_at	*HNRNPA1*	heterogeneous nuclear ribonucleoprotein A1	-1.0	0.50
1424252_at	*HNRPDL*	heterogeneous nuclear ribonucleoprotein D-like	-1.5	0.35
1424802_a_at	*LUC7L3*	LUC7-like 3 (S. cerevisiae)	-1.3	0.41

To be cont'd

Affymetrix ID	Gene symbol	Functional category/ gene description	Signal log ratio	Fold change
1422850_at	*PABPN1*	poly(A) binding protein, nuclear 1	-1.8	0.29
1443282_at	*PRPF38A*	PRP38 pre-mRNA processing factor 38 (yeast) domain containing A	-1.0	0.50
1452869_at	*PRPF38B*	PRP38 pre-mRNA processing factor 38 (yeast) domain containing B	-1.3	0.41
1428172_at	*PRPF39*	PRP39 pre-mRNA processing factor 39 homolog (yeast)	-1.1	0.47
1455358_at	*RBFOX1*	RNA binding protein, fox-1 homolog (C. elegans) 1	-1.1	0.47
1429169_at	*RBM3*	RNA binding motif protein 3	-1.3	0.41
1443033_at	*RBM14*	RNA binding motif protein 14	-1.0	0.50
1443715_at	*RBM24*	RNA binding motif protein 24	-1.2	0.44
1425523_at	*RBM25*	RNA binding motif protein 25	-1.5	0.35
1435391_at	*RBM33*	RNA binding motif protein 33	-1.0	0.50
1426863_at	*RBMX*	RNA binding motif protein, X chromosome	-1.8	0.29
1429504_at	*RNPC3*	RNA-binding region (RNP1, RRM) containing 3	-1.9	0.27
1427988_s_at	*SAFB2*	scaffold attachment factor B2	-1.0	0.50
1427134_at	*SFRS12*	splicing factor, arginine/serine-rich 12	-1.0	0.50
1424452_at	*SLTM*	SAFB-like, transcription modulator	-1.1	0.47
		Membrane transport		
1419758_at	*ABCB1A*	ATP-binding cassette, sub-family B (MDR/TAP), member 1A	1.4	2.64
1443870_at	*ABCC4*	ATP-binding cassette, sub-family C (CFTR/MRP), member 4	1.8	3.48
1456003_a_at	*SLC1A4*	solute carrier family 1 (glutamate/neutral amino acid transporter), member 4	1.0	2.00
1434773_a_at	*SLC2A1*	solute carrier family 2 (facilitated glucose transporter), member 1	1.2	2.30
1425364_a_at	*SLC3A2*	solute carrier family 3 (activators of dibasic and neutral amino acid transport), member 2	1.0	2.00
1443536_at	*SLC7A11*	solute carrier family 7 (cationic amino acid transporter, y+ system), member 11	2.9	7.46
1415802_at	*SLC16A1*	solute carrier family 16 (monocarboxylic acid transporter), member 1	1.1	2.14
1449005_at	*SLC16A3*	solute carrier family 16 (monocarboxylic acid transporter), member 3	1.3	2.46
1417902_at	*SLC19A2*	solute carrier family 19 (thiamine transporter), member 2	1.2	2.30
1450409_a_at	*SLC48A1*	solute carrier family 48 (heme transporter), member 1	1.2	2.30

To be cont'd

Affymetrix ID	Gene symbol	Functional category/ gene description	Signal log ratio	Fold change
1427371_at	*ABCA8A*	ATP-binding cassette, sub-family A (ABC1), member 8A	-1.2	0.44
1435752_s_at	*ABCC9*	ATP-binding cassette, sub-family C (CFTR/MRP), member 9	-1.8	0.29
1436602_x_at	*CACNA1B*	calcium channel, voltage-dependent, N type, alpha 1B subunit	-1.4	0.38
1438109_at	*CLCA5*	chloride channel calcium activated 5	-1.2	0.44
1454768_at	*KCNF1*	potassium voltage-gated channel, subfamily F, member 1	-1.1	0.47
1426633_s_at	*KCTD14*	potassium channel tetramerisation domain containing 14	-1.9	0.27
1435933_at	*SCN2A1*	sodium channel, voltage-gated, type II, alpha 1	-1.3	0.41
1458813_at	*SCN5A*	sodium channel, voltage-gated, type V, alpha	-1.0	0.50
1432798_at	*SLC12A6*	solute carrier family 12 (potassium/chloride transporter), member 6	-1.3	0.41
1457266_at	*SLC38A6*	solute carrier family 38 (sodium-coupled neutral amino acid transporter), member 6	-1.0	0.50
		Signal peptide		
1420380_at	*CCL2*	chemokine (C-C motif) ligand 2	3.6	12.13
1421228_at	*CCL7*	chemokine (C-C motif) ligand 7	2.2	4.59
1419209_at	*CXCL1*	chemokine (C-X-C motif) ligand 1	4.4	21.11
1419728_at	*CXCL5*	chemokine (C-X-C motif) ligand 5	4.3	19.70
1417574_at	*CXCL12*	chemokine (C-X-C motif) ligand 12	1.2	2.30
1449195_s_at	*CXCL16*	chemokine (C-X-C motif) ligand 16	2.5	5.66
1418949_at	*GDF15*	growth differentiation factor 15	1.2	2.30
1421992_a_at	*IGFBP4*	insulin-like growth factor binding protein 4	1.0	2.00
1427747_a_at	*LCN2*	lipocalin 2	2.9	7.46
1451054_at	*ORM1*	orosomucoid 1	3.4	10.56
1425454_a_at	*IL12A*	interleukin 12a	-1.1	0.47
1423915_at	*OLFML2B*	olfactomedin-like 2B	-1.0	0.50
1451031_at	*SFRP4*	secreted frizzled-related protein 4	-1.1	0.47
1450004_at	*TSLP*	thymic stromal lymphopoietin	-2.5	0.18
		Leucine-rich repeat		

To be cont'd

Affymetrix ID	Gene symbol	Functional category/ gene description	Signal log ratio	Fold change
1448421_s_at	*ASPN*	asporin	-2.6	0.16
1435779_at	*CEP110*	centrosomal protein 110	-1.2	0.44
1428427_at	*FBXL2*	F-box and leucine-rich repeat protein 2	-1.2	0.44
1437685_x_at	*FMOD*	fibromodulin	-1.7	0.31
1436218_at	*LGR6*	leucine-rich repeat-containing G protein-coupled receptor 6	-1.7	0.31
1429679_at	*LRRC17*	leucine rich repeat containing 17	-1.1	0.47
1451498_at	*LRRC26*	leucine rich repeat containing 26	-1.1	0.47
1445841_at	*LRRC39*	leucine rich repeat containing 39	-1.3	0.41
1423607_at	*LUM*	lumican	-2.2	0.22
1418745_at	*OMD*	osteomodulin	-2.9	0.13
1416321_s_at	*PRELP*	proline arginine-rich end leucine-rich repeat	-1.3	0.41
		Extracellular structure/ cytoskeleton		
1446326_at	*COL1A2*	collagen, type I, alpha 2	-1.0	0.50
1425476_at	*COL4A5*	collagen, type IV, alpha 5	-1.3	0.41
1424131_at	*COL6A3*	collagen, type VI, alpha 3	-1.4	0.38
1418440_at	*COL8A1*	collagen, type VIII, alpha 1	-1.6	0.33
1434667_at	*COL8A2*	collagen, type VIII, alpha 2	-1.0	0.50
1440911_at	*COL23A1*	collagen, type XXIII, alpha 1	-1.5	0.35
1438540_at	*COL25A1*	collagen, type XXV, alpha 1	-1.3	0.41
1417307_at	*DMD*	Dystrophin, muscular dystrophy	-1.4	0.38
1443745_s_at	*DMP1*	dentin matrix protein 1	-1.4	0.38
1418511_at	*DPT*	dermatopontin	-1.5	0.35
1423915_at	*OLFML2B*	olfactomedin-like 2B	-1.0	0.50
1451041_at	*ROCK2*	Rho-associated coiled-coil containing protein kinase 2	-1.1	0.47
1417860_a_at	*SPON2*	spondin 2, extracellular matrix protein	-1.7	0.31
1438303_at	*TGFB2*	transforming growth factor, beta 2	-1.0	0.50

Global gene expression profile was evaluated in C2C12 cells after treatment with 0.125% (w/v) berry extract for 24 hours. Genes that were changed by greater than 2-fold (signal log ratios ≥ 1 or ≤ -1) upon treatment were regarded as differentially expressed.

Table 23. List of functionally-related genes differentially expressed upon treatment with ethanol in C2C12 myotubes

Affymetrix ID	Gene symbol	Functional category/ gene description	Signal log ratio	Fold change
		Insulin signalling/ glucose metabolism		
1452014_a_at	*IGF1*	insulin-like growth factor 1	0.5	1.41
1418349_at	*HEGFL*	heparin-binding EGF-like growth factor	-0.5	0.71
1458268_s_at	*IGFBP3*	insulin-like growth factor binding protein 3	-0.5	0.71
1453069_at	*PIK3CB*	phosphatidylinositol 3-kinase, catalytic, beta polypeptide	-0.8	0.57
1419447_s_at	*TBC1D1*	TBC1 domain family, member 1	-0.5	0.71
1449334_at	*TIMP3*	tissue inhibitor of metalloproteinase 3	-0.5	0.71
		Muscle metabolism and contraction		
1419621_at	*ANKRD2*	Ankyrin repeat domain-containing protein 2	0.5	1.41
1428722_at	*CKMT2*	creatine kinase, mitochondrial 2	0.9	1.87
1417872_at	*FHL1*	four and a half LIM domains 1	0.6	1.52
1422536_at	*TNNI3*	troponin I, cardiac 3	0.5	1.41
1447713_at	*TPM1A*	tropomyosin 1, alpha	0.5	1.41
1453588_at	*CAR3*	carbonic anhydrase 3	-1.4	0.38
1425153_at	*MYH2*	myosin heavy chain IIa (MyHC-IIa), skeletal muscle, adult, fast-twitch	-0.6	0.66
1448553_at	*MYH7*	myosin heavy chain β (MyHC-β), cardiac muscle	-0.5	0.71
		Transcription factor		
1418174_at	*DBP*	D site albumin promoter binding protein	0.8	1.74
1422870_at	*HOXC4*	homeo box C4	-0.5	0.71
1418932_at	*NFIL3*	nuclear factor, interleukin 3, regulated	-0.6	0.66
1422103_a_at	*STAT5B*	signal transducer and activator of transcription 5B	-0.5	0.71
		Cell cycle		
1448494_at	*GAS1*	growth arrest-specific protein 1	0.5	1.41
1417419_at	*CCND1*	cyclin D1	-0.5	0.71
1434830_at	*MXD1*	MAX dimerisation protein 1	-0.7	0.62
1450337_a_at	*NEK8*	NIMA (never in mitosis gene a)-related expressed kinase 8	-0.5	0.71

To be cont'd

Affymetrix ID	Gene symbol	Functional category/ gene description	Signal log ratio	Fold change
		Protein synthesis		
1437071_at	*EIF1AX*	eukaryotic translation initiation factor 1-alpha, X-linked	-0.5	0.71
1440866_at	*EIF2AK2*	eukaryotic translation initiation factor 2-alpha kinase 2	-0.7	0.62
1455108_at	*EIF4E2*	eukaryotic translation initiation factor 4E member 2	-0.6	0.66
1417978_at	*EIF4E3*	eukaryotic translation initiation factor 4E member 3	-0.6	0.66

Global gene expression profile was evaluated in C2C12 cells after treatment with 300 mM ethanol for 24 hours. Genes that were changed by greater than 1.4-fold (signal log ratios ≥ 0.5 or ≤ -0.5) upon treatment were regarded as differentially expressed.

3.2.5.3 Validation of microarray data with ethanol treatment by qRT-PCR

Validation of the microarray data for C2C12 myotubes treated with ethanol was performed using a qRT-PCR approach. Five differentially expressed (as shown in the microarray) genes which are related to muscle metabolism and contraction, as well as five other genes involved in insulin signalling were selected for validation. These were Ankyrin repeat domain-containing protein 2 (*ANKRD2*), myosin heavy chain IIa, adult, fast-twitch (MyHC-IIa) (*MYH2*), myosin, heavy chain β, cardiac (MyHC-β) (*MYH7*), troponin I type 3 (*TNNI3*) and tropomyosin 1 alpha (*TPM1A*), v-akt murine thymoma viral oncogene homolog 2 (*AKT2*), insulin receptor (*INSR*), insulin receptor substrate 2 (*IRS2*), phosphatidylinositol 3-kinase, catalytic, beta polypeptide (*PIK3CB*) and TBC1 domain family, member 1 (*TBC1D1*).

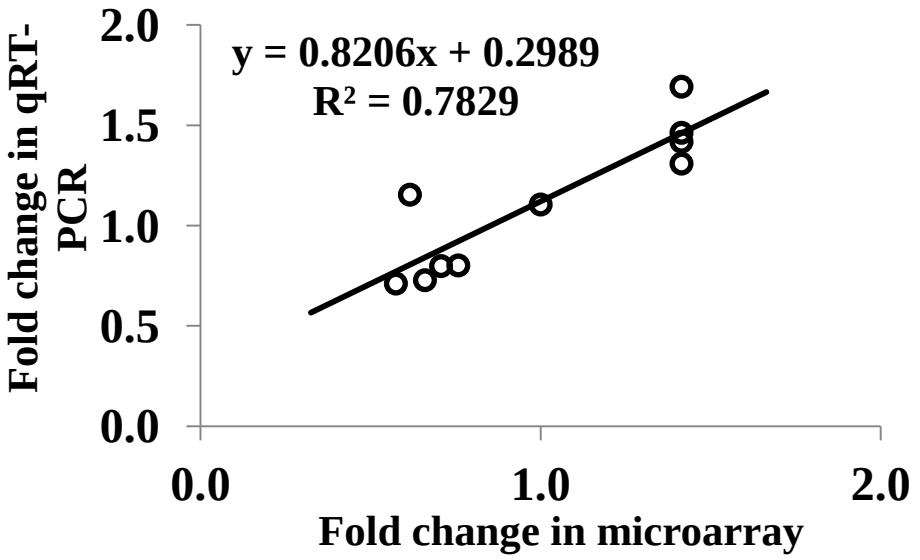

Figure 40. Comparison of fold changes in gene expression as shown in microarray and qRT-PCR

Fold changes in the expression of the ten selected genes for validation as shown in microarray and qRT-PCR were compared. Genes investigated are Ankyrin repeat domain-containing protein 2 (*ANKRD2*), myosin heavy chain IIa, adult, fast-twitch (MyHC-IIa) (*MYH2*), myosin, heavy chain β, cardiac (MyHC-β) (*MYH7*), troponin I type 3 (*TNNI3*) and tropomyosin 1 alpha (*TPM1A*), v-akt murine thymoma viral oncogene homolog 2 (*AKT2*), insulin receptor (*INSR*), insulin receptor substrate 2 (*IRS2*), phosphatidylinositol 3-kinase, catalytic, beta polypeptide (*PIK3CB*) and TBC1 domain family, member 1 (*TBC1D1*). Pearson's correlation coefficient, R= 0.885, P= 0.001.

Pearson's product moment test was performed to determine the correlation between results from microarray and qRT-PCR. As shown in **Figure 40**, fold changes of the expression of the ten selected genes were highly concordant between the two detection methodologies (R= 0.885, P= 0.001).

Fold changes of individual genes were also compared and presented as a bar chart in **Figure 41**. As shown in this figure, fold changes of the gene expression showed similar directions in microarray and qRT-PCR, except for insulin receptor (*INSR*). This can be explained by the very low abundance of *INSR* mRNA in our samples and thus a relatively high background signal effect in the microarray analysis. In fact, the changes of *INSR* expression did not reach significance in both microarray (MAS5.0 algorithm) and qRT-PCR (see **Table 24**). Furthermore, although the microarray showed a down-regulation for the gene of myosin heavy chain IIa (*MYH2*) and an up-regulation for troponin I type3 (*TNNI3*), the changes did not reach statistical significance in qRT-PCR (see **Figure 41** and **Table 24**). This was probably due to the use of pooled RNA samples in the microarray study and thus any variations between samples were not taken into consideration. In contrast, a significant up-regulation of insulin receptor substrate 2 (*IRS2*) was reported in the qRT-PCR but not in the microarray. This was not surprising as qRT-PCR is generally regarded as a more sensitive methodology for measuring changes in gene expression (Klein, 2002). Otherwise, we confirmed that in C2C12 myotubes, upon treatment with ethanol, the gene expressions of Ankyrin repeat domain-containing protein 2 (*ANKRD2*) and tropomyosin 1 alpha (*TPM1A*) were increased, whereas the gene expressions of myosin, heavy chain β, cardiac (*MYH7*), phosphatidylinositol 3-kinase, catalytic, beta polypeptide (*PIK3CB*) and TBC1 domain family, member 1 (*TBC1D1*) were decreased (see **Figure 41** and **Table 24**).

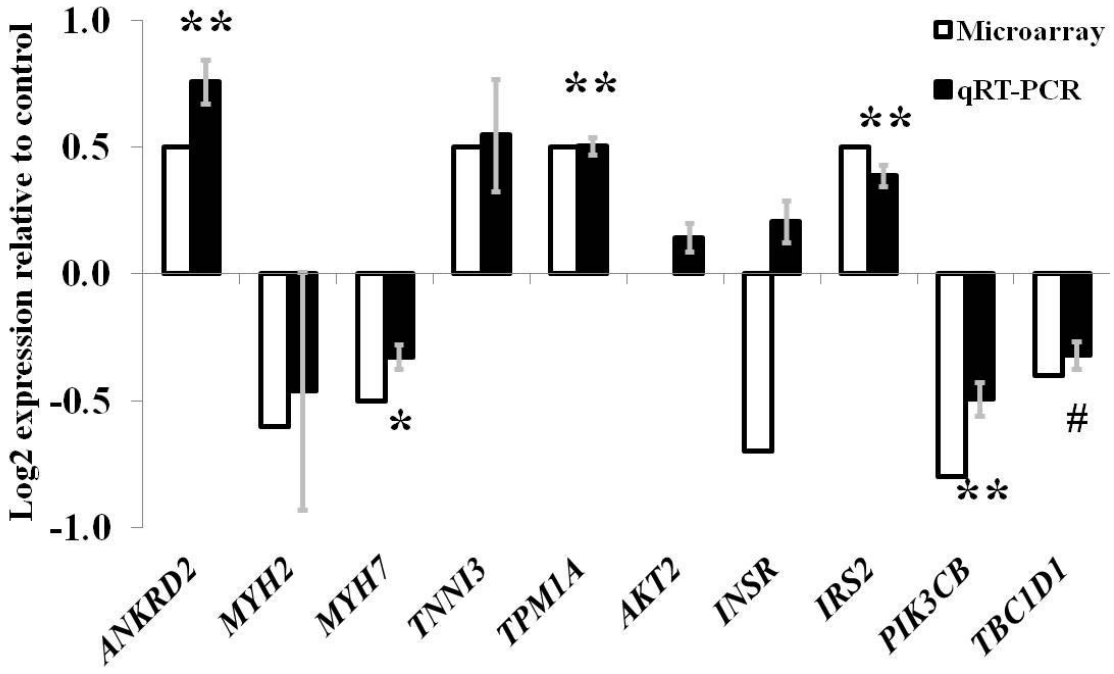

Figure 41. Validation of microarray data for ten selected genes by qRT-PCR

Five muscle metabolism and contraction-related genes, Ankyrin repeat domain-containing protein 2 (*ANKRD2*), myosin heavy chain IIa, adult, fast-twitch (MyHC-IIa) (*MYH2*), myosin, heavy chain β, cardiac (*MYH7*), troponin I type 3 (*TNNI3*) and tropomyosin 1 alpha (*TPM1A*), and five genes involving in insulin signalling, v-akt murine thymoma viral oncogene homolog 2 (*AKT2*), insulin receptor (*INSR*), insulin receptor substrate 2 (*IRS2*), phosphatidylinositol 3-kinase, catalytic, beta polypeptide (*PIK3CB*) and TBC1 domain family, member 1 (*TBC1D1*),were selected for validation by qRT-PCR. Microarray: data are presented as mean; qRT-PCR: data are presented as mean ± SEM (n= 9). $*P< 0.01$, $^{\#}P< 0.005$, $**P< 0.001$ compared with control in qRT-PCR.

Table 24. Changes in the expression of the ten selected genes in the microarray and in qRT-PCR

Gene	Change call in microarray	Change in qRT-PCR (*P*-value)
Muscle development and contraction		
ANKRD2	Increase	Increase ($P< 0.001$)
MYH2	Decrease	No change ($P= 0.492$)
MYH7	Decrease	Decrease ($P= 0.006$)
TNNI3	Increase	No change ($P= 0.068$)
TPM1A	Increase	Increase ($P< 0.001$)
Insulin signalling		
AKT2	No change	No change ($P= 0.103$)
INSR	No change	No change ($P= 0.200$)
IRS2	No change	Increase ($P< 0.001$)
PIK3CB	Decrease	Decrease ($P< 0.001$)
TBC1D1	Decrease	Decrease ($P= 0.002$)

Due to time limitations for the project for this thesis, qRT-PCR validation for the microarray data of berry extract-treated C2C12 muscle cells could not be performed. However, the microarray studies for berry extract and ethanol treatments were carried out simultaneously and using the same batch of materials, most importantly the microarray chips. Given the high concordance in the changes of gene expression between microarray and qRT-PCR for the ethanol-treated cells (see **Figure 40**, **Figure 41** and **Table 24**), we are confident that the microarray data for berry extract treatment were valid.

4 Discussion

A summary of the main findings on the effects of berry extract and ethanol on glucose transport is presented in **Table 25**. The ensuing section will then begin with an account of the acute and chronic effects of berry extract and related polyphenols on glucose transport in intestinal epithelial Caco-2 cells. This is followed by a scientific discussion into the cellular effects of chronic exposure to berry extract on glucose transport in C2C12 muscle cells. I will then discuss the results obtained from microarray studies in C2C12 muscle cells following berry either extract or ethanol treatments.

Table 25. A summary of the cellular effects of berry extract and ethanol on glucose uptake in Caco-2 intestnal cells and C2C12 muscle cells

	Caco-2 intestinal cells	**C2C12 muscle cells**
Acute studies	Berry extract and the related flavonoids- quercetin, cyanidin and their glycosides- acutely inhibited glucose uptake by directly interacting with glucose transporters.	Not carried out.
Chronic studies	Berry extract down-regulated the gene expression of the two main intestinal glucose transporters, GLUT2 and SGLT1.	At the highest dose tested (0.125% (w/v)), berry extract moderately up-regulated the gene expression of GLUT1.
	Berry extract also up-regulated the expression of the *let*-7a miRNA, a potential negative regulator of GLUT2.	At the functional level, berry extract did not induce any changes in glucose uptake in C2C12 myotubes, neither in basal nor insulin-stimulated conditions.
	At the functional level, berry extract did not alter the initial rate of glucose uptake in Caco-2 cells.	At high doses, ethanol moderately down-regulated the gene expression of the GLUT4 transporter.

4.1 Acute effects of berry extract and polyphenols on glucose uptake in the intestinal Caco-2 cells

Data obtained from the acute studies indicated that berry extract significantly reduced glucose uptake into Caco-2 cells (**Figure 16**, page 95). Comparing results in sodium-containing and sodium-free conditions, it is suggested that berry extract had similar inhibitory effects on transport through the facilitative glucose transporters (GLUTs) and sodium-dependent glucose transporter (SGLT1). Cyanidin, one of the predominant anthocyanidins in the berry extract, and two of its glycosides, 3-*O*-glucoside and 3-*O*-rutinoside also showed an inhibitory effect on glucose uptake in Caco-2 cells (**Figure 17**, page 97). Anthocyanins occur naturally in foods mainly attached to sugar moieties (Zhang et al., 2004). Our findings suggested that the glucose uptake-inhibitory activity of berry extract is attributed in part to its constituent anthocyanins such as cyanidin-3-*O*-glucoside and cyanidin-3-*O*-rutinoside.

Data in the scientific literature have shown that a wide range of polyphenols such as phloridzin, quercetin and its glucosides, catechins, caffeic acid and pelargonidin glucoside have inhibitory effects on glucose uptake in Caco-2 cells or related intestinal transport systems (Johnston et al., 2005, Chen et al., 2007, Manzano and Williamson, 2010). Our studies showed similar effects of an anthocyanin-rich berry extract on intestinal glucose uptake in a Caco-2 cell model and suggested that cyanidin glycosides may contribute to its inhibition. This is based on the observation that cyanidin glycosides markedly reduced glucose uptake.

The importance of the inhibitory effects of berry extract on intestinal glucose transport does not seem to be triggering a reduction in the total amount of glucose absorbed. This is evident in previous studies by other groups which showed no

difference in the areas under postprandial plasma glucose curves after consumption of apple juice, coffee, berry puree or cranberry juice, all of which are rich in polyphenols (Johnston et al., 2002, Johnston et al., 2003, Wilson et al., 2008, Törrönen et al., 2010, Törrönen et al., 2012b). Instead, the attenuation of the rate of glucose absorption in the proximal jejunum, where the absorptive capacity for glucose is maximal (Mcmichael, 1971), could extend the length of intestinal lumen exposed to glucose, leading to delayed glucose absorption and reduced postprandial glycaemic excursions (see **Figure 42**).

Increased exposure of the distal small intestine to glucose as a result of delayed glucose absorption further stimulates the release of the incretin, glucagon-like peptide-1 (GLP-1), from intestinal endocrine L-cells located mainly in the distal jejunum and ileum (Eissele et al., 1992). This enhancement of GLP-1 secretion could be of particular significance in type 2 diabetic patients as postprandial secretion of GLP-1 is impaired in diabetic conditions, whereas the responsiveness of pancreatic β-cells to GLP-1 is preserved (Vaag et al., 1996, Toft-Nielsen et al., 2001). By promoting the release of GLP-1, the inhibition of proximal intestinal glucose absorption does not only elicit a bigger postprandial insulin response (Mojsov et al., 1987), but also inhibits glucagon secretion, suppresses intestinal motility and slows gastric emptying (Willms et al., 1996, Heller et al., 1997). The abovementioned benefits were consistent with improved glucose tolerance in Goto-Kakizaki type 2 diabetic rats that underwent duodenal-jejunal bypass that excludes the proximal intestine (Rubino et al., 2006).

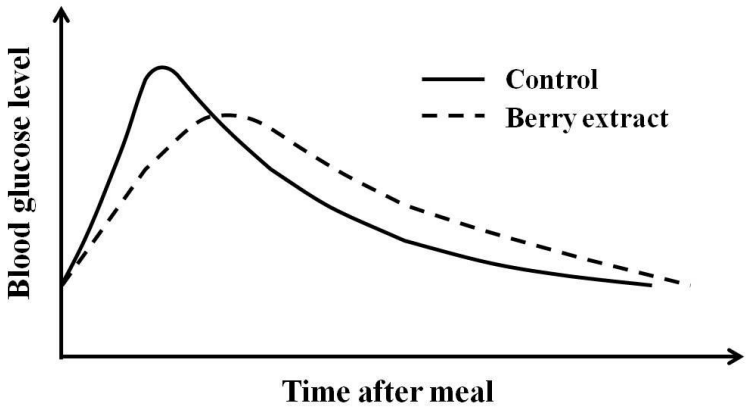

Figure 42. Predicted effect of berry extract consumption on postprandial blood glucose profile

The graph was drawn based on the results from Johnston et al. (2002) and Törrönen et al. (2012b).

4.2 Effects of berry extract and polyphenols on glucose transporter expression in the intestinal Caco-2 cells

Previous studies in the literature mainly focused on the inhibitory effects of polyphenols and food extracts on glucose transport via direct interaction with glucose transporters. We also evaluated the potencies of berry extract and polyphenols to regulate glucose transport in Caco-2 cells through modulations of glucose transporter expression after prolonged incubation (16 hours).

Intriguingly, in our studies, both berry extract and quercetin triggered biphasic changes in the gene expression of GLUT2 with increasing concentrations, i.e. an up-regulation at low concentration but down-regulation at higher concentrations (see **Figure 20**, page 101 and **Figure 23**, page 105). This compares with the expression of SGLT1 which was reduced only at the highest doses of berry extract, but also at all tested concentrations of quercetin. At the highest concentration of berry extract (0.125% (w/v)), the inhibitory effects on the gene expressions of GLUT2 and SGLT1 were evident as early as 4 hours after treatment (see **Figure 22**, page 104). Most

prominent down-regulation of GLUT2 and SGLT1 mRNA levels by 100 μM of quercetin was observed after 12-16 hours of treatment (see **Figure 24**, page 106).

Faria et al. (2009) reported an increase in GLUT2 gene expression in Caco-2 cells after a 96-hour pre-treatment with an anthocyanin-rich extract obtained from red grape skin. The authors suggested that the augmented GLUT2 expression is involved in the enhanced absorption of the anthocyanins and hence their bioavailability after chronic exposure (Faria et al., 2009). Conversely, a Canadian research group has demonstrated reductions in the protein expression of GLUT2 and SGLT1 after incubation of Caco-2 cells with extracts of native anti-diabetic medicinal plants (Nistor Baldea et al., 2010). Phytochemical characterisations have shown that these extracts are rich in flavonoids such as quercetin glycosides (Spoor et al., 2006, Harbilas et al., 2009). It has also been shown that feeding streptozotocin-induced diabetic mice with the apple flavonoid, phloridzin, reversed the streptozotocin-stimulated expression of SGLT1 in the small intestine (Masumoto et al., 2009).

The discrepancies in the effect of flavonoids on the gene expression of intestinal glucose transporters are not fully understood. One possible explanation is that multiple mechanisms are involved in these modulatory effects and different concentrations of flavonoids are required to reach the thresholds to activate individual pathways. In fact, we observed biphasic dose responses in the gene expression of glucose transporters in Caco-2 cells exposed to berry extract or quercetin. This is not unusual, since similar biphasic cellular changes have also been reported in various *in vitro* cell systems in response to a range of quercetin concentrations (Van Der Woude et al., 2005, Santini et al., 2009).

Elevated protein levels of GLUT2 and SGLT1 have been observed in the duodenum of type 2 diabetic patients (Dyer et al., 2002). Also, in about 75% of morbidly obese human subjects, GLUT2 was detected at the apical membrane in jejunal enterocytes in the fasting state, in contrast to its basolateral-only location in lean subjects (Ait-Omar et al., 2011). Together, these aberrations lead to enhanced intestinal glucose absorption, hence provoking increased postprandial glycaemic excursions. Our chronic studies demonstrated that high doses of berry extract down-regulated the gene expression of the two major intestinal glucose transporters, GLUT2 and SGLT1. These findings suggested that consumption of anthocyanin-enriched berry extract might help ameliorate the impairment of glycaemic control in diabetic conditions by reversing the up-regulated expressions of GLUT2 and SGLT1.

4.3 Chronic effects of berry extract and polyphenols on glucose uptake in the intestinal Caco-2 cells

Given the dramatic down-regulation of GLUT2 and SGLT1 gene expression by the highest concentrations of berry extract and quercetin, we would expect a reduction in glucose uptake under the same treatment conditions. However, surprisingly, we did not observe any changes in glucose uptake into Caco-2 cells after prolonged pre-incubation with berry extract or quercetin, nor with the well-established glucose transporter inhibitors, phloretin and phloridzin (see **Figure 25**, page 107 and **Figure 26**, page 108).

The lack of changes at the functional level could be due to the high abundance of GLUT1 and GLUT3 transporters in our Caco-2 subclone. In normal tissue, mRNA levels of GLUT1 and GLUT3 are negligible in the small intestine (Yano et al., 1991, Yoshikawa et al., 2011). However, up-regulation of GLUT1 and GLUT3 expression occurs in the transformation process, thus, elevation of GLUT1 and GLUT3 proteins

has been observed in many cancers (Macheda et al., 2005). Due to the carcinoma origin of Caco-2 cells, constituent and measurable levels of GLUT1 and GLUT3 mRNAs are expected. Mahraoui et al. (1994a) have reported a decline in the mRNA levels of GLUT1 and GLUT3 but an increase for GLUT2 and SGLT1 in the Caco-2/TC7 subclone during differentiation. Nevertheless, several groups (as well as the present studies for this thesis, see **Table 17**, page 92) have reported low or negligible expressions of GLUT2 and SGLT1 compared to that of GLUT1 and GLUT3 in differentiated Caco-2 cells (Sun et al., 2002, Landowski et al., 2004, Dihal et al., 2007, Hayeshi et al., 2008).

We showed that the gene expression of GLUT1 was not altered by any of the concentrations of berry extract tested, whereas that of GLUT3 was reduced only at the highest concentration of 0.125% (w/v) (see **Figure 21**, page 103). GLUT3 is a glucose transporter with a particular high substrate affinity, as reflected by the distinct kinetics compared to GLUT2 (K_m values for deoxyglucose transport of around 1.4 and 11.2 mM, respectively) (Arbuckle et al., 1996). The high abundance and affinity of GLUT3 could therefore mask any changes in glucose uptake attributed to perturbations in the expression of GLUT2 or SGLT1. Furthermore, the rate of carrier-mediated glucose transport into the enterocytes also depends on the level of glucose transporters located at the apical membrane. The distribution of glucose transporters between intracellular compartments and the apical membrane is tightly regulated post-translationally (Kipp et al., 2003, Kellett and Brot-Laroche, 2005). Therefore, glucose uptake might not directly reflect the changes in the gene expression of glucose transporters.

4.4 Global miRNA expression in response to berry extract in the intestinal Caco-2 cells

As mentioned in the Introduction, miRNAs are believed to control the expression of over half to two-thirds of all protein-coding genes (Fabian et al., 2010) and thus have arisen to become important cellular molecules that are involved in numerous integral biological processes. In fact, they have been implicated in the control of glucose homeostasis and the pathogenesis of diabetes (Tang et al., 2008, Frost and Olson, 2011).

Microarrays offer a convenient tool for miRNA expression profiling to quickly and simultaneously determine aberration in the relative expression levels of large numbers of miRNAs in normal cellular and pathophysiological processes. However, due to the short length of miRNAs (~22 nucleotides) and the high similarity in sequences between different miRNA species, the sensitivity and specificity of such hybridisation-based profiling approach have faced technical and practical challenges.

In light of these technical challenges, several studies have evaluated the reliability and reproducibility of the use of microarrays for miRNA expression measurements. Git et al. (2010) reported a high variability in the reproducibility of hybridisation and consistency of present/ absent calls between several miRNA microarray platforms. Comparisons between the performances of microarray and PCR (the latter widely regarded as the "gold standard" in the detection and quantitation of gene expression) showed generally good correlations (Ach et al., 2008, Git et al., 2010). However, both of the abovementioned groups demonstrated that a small number of miRNAs showed particular poor correlations between the two approaches (Ach et al., 2008, Git et al., 2010). A review on miRNA profiling methodologies by Pritchard et al.

(2012) warned that microarrays have a restricted linear range of quantification and poor specificity for miRNAs closely related in sequence.

In the present study for this thesis, the qRT-PCR results validated only three out of the five selected miRNAs, while the changes in the expression of *let-7f* and *miR-106b* showed opposite directions in qRT-PCR and microarray (see **Figure 27**, page 112). This was likely attributed to the high similarity in the sequences between several miRNA species. For example, *let-7d* and *let-7f* differ from *let-7a* by only one single nucleotide (all of which are encoded by a single genomic cluster on chromosome 13) (see **Figure 43**).

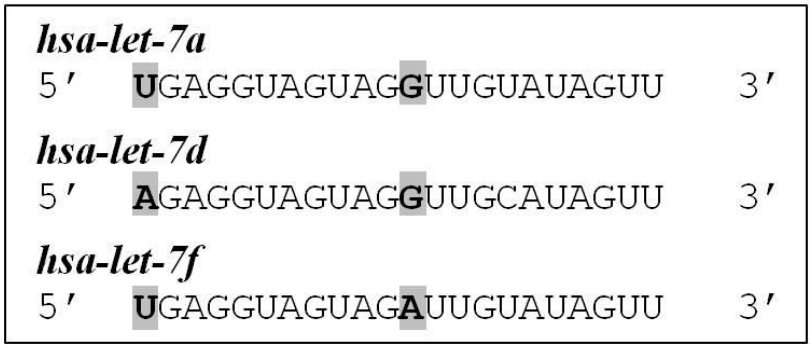

Figure 43. Sequences of the miRNAs, *let-7a*, *let-7d* and *let-7f*

Sequences of the *let-7* family showed high similarity. Differences in the nucleotide are highlighted in grey. miRNA sequences were obtained from the miRNA database, miRBase (http://www.mirbase.org/).

We reported an up-regulation of the miRNA *let-7a* in Caco-2 cells upon treatment with berry extract. Numerous studies have demonstrated the important role of the *let-7* miRNA family in growth and development and have shown that the aberration of *let-7a* expression is implicated in the development of cancers (Johnson et al., 2005, Akao et al., 2006, Johnson et al., 2007, He et al., 2009, Yang et al., 2011). More recently, it was revealed that *let-7* miRNAs are also involved in glucose metabolism and insulin sensitivity (Frost and Olson, 2011, Zhu et al., 2011). Using

the miRNA target prediction algorithm (*TargetScanHuman version 6.2*; http://www.targetscan.org/), we found that *let-7a* is predicted to target the mRNA of GLUT2. Our findings proposed a role of *let-7a* in the down-regulation of GLUT2 gene expression by berry extract but further studies using miRNA transfection techniques will be required to substantiate this.

4.5 Effects of berry extract on glucose transport in C2C12 myotubes

The use of natural foods and herbal medicines as an alternative to pharmacological agents for therapies of diabetes has recently attracted much attention. Specifically, there is a large body of literature that have reported modulatory effects of polyphenols on glucose transport in skeletal muscles, the principal site of postprandial glucose disposal.

In cultured muscle cells, polyphenol-rich extracts of bitter melon (*Momordica charantia*) or root of Indian shot (*Canna indica* L.) have been shown to stimulate glucose uptake (Purintrapiban et al., 2006, Kumar et al., 2009). The increase in glucose uptake induced by bitter melon extracts was in parallel with an up-regulation of GLUT4 expression that required *de novo* protein synthesis (Kumar et al., 2009). On the other hand, extracts of root of Indian shot was demonstrated to enhance the *de novo* synthesis of GLUT1 protein, as well as to promote the levels of GLUT1 and GLUT4 located at the plasma membrane (Purintrapiban et al., 2006).

In obese diabetic *ob*/*ob* mice, treatment with the ubiquitous flavonoid quercetin improved hyperglycemia and insulin resistance (Anhe et al., 2012). These effects were at least in part due to an increase in GLUT4 expression in muscle (Anhe et al., 2012). Similar improvements in plasma glucose and glucose tolerance was also

observed in *ob*/*ob* mice fed with a citrus flavonoid, nobiletin (Lee et al., 2010b). However, in the latter study, an up-regulation of GLUT1 expression and an increase in the level of GLUT4 proteins on the plasma membrane in muscle were observed (Lee et al., 2010b).

Tea extracts have also been shown to possess anti-diabetic effects in mice fed with high-fat or high-fructose diets (Cao et al., 2007, Nishiumi et al., 2010), both of which can induce hyperglycemia and insulin resistance (Luo et al., 1998). In both models, restoration of the diets-induced reduction of GLUT4 expression in muscles was observed (Cao et al., 2007, Nishiumi et al., 2010).

In the present study, the dose-response investigation of berry extract on gene expression of glucose transporter in C2C12 muscle cells demonstrated no changes in GLUT4 mRNA levels across all concentrations tested. In contrast, while the lowest dose of 0.008% (w/v) suppressed GLUT1 expression, the highest dose (0.125% (w/v)) increased it (see **Figure 35**, page 120).

Muscle is the insulin-sensitive tissue responsible for the majority of insulin-mediated glucose disposal. It was estimated that glucose uptake into skeletal muscles accounted for only approximately 20% of whole body glucose uptake in the basal state (Baron et al., 1988). However, during hyperinsulinemia, insulin-mediated glucose uptake in skeletal muscle represented 75% and 95% of whole body glucose disposal, at euglycemia and hyperglycemia, respectively (Baron et al., 1988). Thus, defects of glucose uptake into muscle in response to insulin action represent a characteristic feature of type 2 diabetes (Defronzo et al., 1985).

In an attempt to evaluate the effects of berry extract on glucose uptake in muscle cells under insulin-stimulated conditions, we chronically incubated differentiating C2C12 cells with 100 nM insulin, starting from 3 days before and during exposure to berry extract. Chronic exposure of cultured muscle cells to insulin has been shown to lead to the development of insulin resistance (Kumar and Dey, 2003). This occurs through prolonged insulin-induced stimulation of JNK and PI3K pathways leading to phosphorylation of IRS-1 at serine^{318} (Mussig et al., 2005). Serine^{318} phosphorylation of IRS-1 has been found to be associated with a reduction of IR/IRS-1 binding and IRS-1 tyrosine phosphorylation (Pederson et al., 2001). It also induced proteosomal degradation of IRS-1 and a reduction in the acute insulin-elicited translocation of GLUT4 (Sun et al., 1999, Huang et al., 2002). Altogether, these changes in IRS-1 and GLUT4 lead to an impaired insulin signalling.

We showed that in C2C12 myotubes, chronic incubation with insulin alone induced a striking 2.7-fold up-regulation of the gene expression of GLUT4. Conversely, a 22% down-regulation in the gene expression of GLUT1 was observed (P= 0.005, Student's t-test; not significant in one-way ANOVA) (see **Figure 37**, page 122). These changes were reflected at the functional level, as glucose uptake was only stimulated by acute exposure (30 minutes) to insulin in C2C12 myotubes pre-treated with insulin (see **Figure 38**, page 124). Our observations opposed previous findings by other groups mentioned above, i.e. chronic insulin exposure induced insulin resistance in cultured muscle cells. Instead, 3-day insulin exposure in C2C12 myotubes led to a development of insulin sensitivity.

We do not know why this occurred but interestingly, in contrast to studies reporting development of insulin insensitivity in muscle cells chronically incubated with

insulin, others suggested that insulin promotes mitogenesis and myogenesis (Mandel and Pearson, 1974, Ewton and Florini, 1981, Sasaoka et al., 1994, Milasincic et al., 1996). This is in accordance with our observations that insulin pre-treatment promoted differentiation in C2C12 cells, determined from histological evidence of a more extensive development of multinucleated myotubes (see **Figure 36**, page 121). Insulin pre-treated cells also appeared to cause changes in intermediary metabolism (personal observations showed that the phenol red pH indicator in culture medium changed colour more rapidly, presumably due to higher rate of acidic metabolites production). Furthermore, both RNA and protein yields were higher in insulin pre-treated cells (data not shown).

Given that our insulin pre-treatment regimen promoted differentiation of C2C12 cells and sensitised the myotubes to insulin action on glucose uptake, we showed that berry extract did not alter glucose uptake in insulin-sensitive C2C12 cells. Although berry extract elicited a 2.4-fold increase in the mRNA level GLUT1 in insulin pre-treated C2C12 cells, the basal glucose uptake did not alter following berry extract treatment. Berry extract did not have any effects on insulin-mediated glucose uptake.

4.5.1 Possible involvement of signalling pathways in the regulation of glucose uptake in C2C12 myotubes

In our studies, we aimed to evaluate the chronic effects of berry extract on glucose uptake in C2C12 muscle cells via the regulation of glucose transporter gene expression. Thus, our glucose uptake studies in C2C12 muscle cells were carried out in the absence of berry extract. Furthermore, C2C12 cells were pre-incubated in berry extract-free buffer at room temperature for 15 minutes prior to the uptake experiments. Under these conditions, where post-translational modifications such as

phosphorylation might have completely or partially lost, we found that berry extract had no effect on either basal or insulin-stimulated glucose uptake in C2C12 cells. However, *in vitro* and *in vivo* studies by other research groups have shown that various flavonoids and flavonoid-rich food extracts modified glucose uptake in skeletal muscle by modulating insulin-dependent or insulin-independent signalling pathways. These studies will be discussed in the following section.

4.5.1.1 The insulin signalling pathways in the C2C12 myotubes

Intravenous injection of the flavonoid myricetin improved insulin sensitivity in high-fructose chow-fed rats (Liu et al., 2007). This was in part attributed to an increased basal as well as insulin-stimulated phosphorylation of IR, IRS-1 and AKT. Furthermore, myricetin treatment also improved the defective action of insulin on the translocation of glucose transporter 4 (GLUT4) in insulin-resistant soleus muscle (Liu et al., 2007).

Kaempferol 3-neohesperidoside was demonstrated to have a stimulatory effect on glucose uptake in rat soleus muscle (Zanatta et al., 2008). Insulin did not show a synergistic effect with the abovementioned flavonoid, while pre-treatment with inhibitors of PKC or PI3K blocked its stimulatory effect. On the other hand, inhibitors of MEK or protein synthesis did not have any effect (Zanatta et al., 2008). These results suggested that kaempferol 3-neohesperidoside increases glucose uptake in rat soleus muscle, at least in part, via the PKC and PI3K pathways. Moreover, the same research group recently showed that another glycoside of kaempferol, kaempferol 3,7-dirhamnoside, also stimulated glucose uptake in rat soleus muscle, and was completely blocked by inhibitors of insulin receptor tyrosine kinase, PI3K

and atypical PKC, as well as a microtubule-depolymerising agent that prevents GLUT4 translocation (Cazarolli et al., 2013).

4.5.1.2 The AMPK pathway in the C2C12 myotubes

In muscle cell models, either C2C12 or L6 myotubes, treatments with either the citrus flavonoids, naringenin and tangeretin (Zygmunt et al., 2010, Kim et al., 2012), a flavonoid-rich fermented blueberry juice (Tri et al., 2007), an ethanol extract of lingonberris, or its constituent polyphenols, the quercetin aglycone, quercetin-3-*O*-glucoside or quercetin-3-*O*-galactoside (Eid et al., 2010), stimulated glucose uptake. The increase in glucose uptake was accompanied by increased phosphorylation of AMPK but not AKT, suggesting the involvement of the insulin-independent AMPK pathway (Tri et al., 2007, Eid et al., 2010, Zygmunt et al., 2010, Kim et al., 2012).

In KKA^y obese and diabetic mice, supplementation of a bilberry extract rich in anthocyanins ameliorated plasma glucose level and restored insulin sensitivity (Takikawa et al., 2010). These changes were accompanied by increases in total AMPKα in white adipose tissue (WAT) and phosphorylation of AMPKα at Thr-172 in WAT as well as skeletal muscle and liver. Protein expression of GLUT4 was also found to be up-regulated (Takikawa et al., 2010).

Our studies focused on the chronic effects of berry extract on glucose transporter expression in C2C12 muscle cells and demonstrated that berry extract did not elicit any effect on glucose uptake in C2C12 cells following 24-hour pre-treatment (see **Figure 38**, page 124). Based on the studies discussed above, we postulated that berry extract might exert an acute effect on glucose transport in skeletal muscles by

inducing the translocation of GLUT4 transporters to the plasma membrane. This might occur via modulations of insulin-dependent or insulin-independent signalling pathways. However, time limitations for the project precluded us from further investigating the role of signalling pathways in the effect of berry flavonoids on glucose uptake in muscle. Future works will be required to test these hypotheses. These include the examinations of GLUT4 subcellular locations and evaluations of the phosphorylation states (activities) of various molecules involved in insulin-dependent or insulin-independent signalling pathways in response to berry extract.

4.6 Global gene expression profiling of C2C12 myotubes in response to berry extract

Our microarray data demonstrated that berry extract at 0.125% (w/v) triggered differential expression of genes involved in multiple cellular functions. In the following discussion, I will focus on the genes implicated in glutathione metabolism, transcriptional regulation, nucleic acid binding/ processing, membrane transport and extracellular structure/ cytoskeleton.

4.6.1 Glutathione metabolism

Glutathione-S-transferases (GSTs) are a family of phase II xenobiotic-metabolising enzymes that modify xenobiotic compounds allowing their subsequent excretion (Douglas, 2006). Their ability to detoxify xenobiotics gives rise to their pivotal role in protecting cells against oxidative stress.

Numerous studies have reported induction of the expressions or activities of GSTs by flavonoids (Moon et al., 2006). For example, oral feeding of flavonoid-rich green tea leaves to rats resulted in a significant increase in the activities of GST in the liver as compared to the control group (Lin et al., 1998), whereas bilberry anthocyanins up-

regulated the expression of GST-pi in cultured human retinal pigment epithelial cells (Milbury et al., 2007). Nevertheless, other groups also demonstrated an inhibition of GST activity by flavonoids such as quercetin and kaempferol (Sahu and Gray, 1996, Van Zanden et al., 2004). Our microarray data showed that berry extract induced an up-regulation of a handful of different GST classes. These include the GST-alpha 1 to 4 (GSTA1-4) and GST-mu 1 (GSTM1) from the cytosolic superfamily, and two microsomal isoforms (MGST1 and 2). The MAS5.0 algorithm also identified an up-regulation for three more isoforms, namely GST-omega 1 (GSTO1), GST-pi 2 (GSTP2) and microsomal GST 3 (MGST3) (fold changes are 1.7, 1.6 and 1.3, respectively), although the changes did not reach the 2-fold threshold.

The activity of GSTs depends on a steady supply of GSH within the cell. In this regard, we also revealed an up-regulation of two enzymes that regulate the cellular level of GSH. These are glutamate cysteine ligase (GCL), the first enzyme in the GSH biosynthesis pathway, and glucose-6-phosphate dehydrogenase (G6PD), a cytosolic enzyme that maintains the level of the co-enzyme nicotinamide adenine dinucleotide phosphate (NADPH), which in turn maintains the level of GSH.

Perturbations in the GSH-dependent xenobiotic metabolism have been demonstrated in diabetes (Raza et al., 2000, Fujita et al., 2001, Yalin et al., 2007). There is also clear evidence that defects in GSH metabolism is implicated in the development of cancers (Schipper et al., 1997, Locigno and Castronovo, 2001). Our finding provides preliminary evidence that flavonoid-rich berry extract may increase cellular antioxidant defence and may serve as a dietary strategy for the prevention and treatment of diabetes and cancers.

4.6.2 Transcriptional regulation

Thirty six genes differentially expressed as a result of berry extract treatment were grouped into the category of transcriptional regulation. This implies that berry extract elicited a wide range of cellular changes through the regulation of genes at the transcriptional level.

4.6.2.1 CCAAT/ enhancer-binding proteins

Among the 36 genes involved in transcriptional regulation, the CCAAT/ enhancer-binding proteins (C/EBPs) are of particular interest due to their regulation by antioxidants. Three members from this family, i.e. the –alpha, -beta and –delta isoforms, were up-regulated by berry extract. The C/EBP-beta transcription factor has been shown to mediate the modulation of gene expression of antioxidant enzymes by flavonoids such as genistein (Wiegand et al., 2009). Interestingly, the C/EBP response elements have been found on the regulatory promoter regions of a few phase II enzymes (Kang et al., 2003). These include glutamate cysteine ligase (GCL) and GST-alpha (GSTA) (mentioned above in the Glutathione metabolism section), as well as NAD(P)H dehydrogenase, quinone 1 (NQO-1) and heme oxygenase 1 (HMOX-1) (Kang et al., 2003). All of these phase II enzymes were up-regulated at the mRNA level by berry extract. Therefore, C/EBPs may serve as common transcriptional factors that co-ordinate the induction of antioxidant genes by berry extract.

4.6.2.2 p53 tumour suppressor protein and related genes

The p53 protein plays an important role in the regulation of cell cycle and functions as a tumour suppressor (Braithwaite et al., 1987, Finlay et al., 1989). Studies have demonstrated that flavonoids can induce G2/M cell cycle arrest and apoptosis in both non-tumour and cancer cell lines via a p53-dependent mechanism (Plaumann et al.,

1996, Kook et al., 2007, Vidya Priyadarsini et al., 2010). In this study, berry extract elevated the gene expression of p53. In addition to the changes in the expression of p53 itself, we also observed an up-regulation of activating transcription factor 3 (ATF3), which encodes for a protein involved in stabilisation of p53 (Yan and Boyd, 2006). ATF3 and fos-like antigen 2 (FOSL2), the latter which was also up-regulated following berry treatment, are components of the heterodimeric transcription factor, activator protein 1 (AP-1) (Hai and Curran, 1991). It is proposed that there is a functional link between AP-1 and p53 as both molecules are involved in the control of cell survival in response to extracellular stimuli (Kirch et al., 1999, Ameyar et al., 2003, Hess et al., 2004). The authors suggested that interactions between these two proteins play an essential role in cell fate decisions (Kirch et al., 1999, Ameyar et al., 2003, Hess et al., 2004).

Altogether, the changes in the expression of p53 and p53-related genes showed that berry extract seemed to favour an activation of the p53 pathway, which would likely initiate cellular changes that ultimately lead to cell cycle arrest or cell death. However, it is worth mentioning that p53 is also subjected to complex regulation by undergoing numerous post-translational modifications such as phosphorylation and ubiquitination (Laptenko and Prives, 2006).

4.6.3 Nucleic acid binding/ processing

Remarkably, berry extract elicited a down-regulation of the expression of all genes involved in nucleic acid binding/ processing, except one, namely the RNA binding protein gene with multiple splicing (RBPMS).

4.6.3.1 RNA binding motif proteins

RNA binding motif proteins (RBPs) are a large family of proteins that associate with RNAs to form ribonucleoprotein (RNP) complexes. Formation of RNP complexes allows extensive post-transcriptional regulation of gene expression by alternative splicing, RNA modification, polyadenylation, mRNA localisation, etc (Glisovic et al., 2008). Our data showed down-regulation of 8 genes from this family but up-regulation of RBPMS in cells treated with berry extract. Since RBPs possess different modes of action and diverse RNA-sequence specificities and affinities, the down-regulation of multiple RBPs by berry extract might represent an inhibition of the processing of a variety of pre-mRNAs/ mRNAs.

4.6.4 Membrane transport

Differentially expressed genes involved in membrane transport include four genes encoding ATP-binding cassette transporters.

4.6.4.1 ATP-binding cassette transporters

Among the four ATP-binding cassette (ABC) transporters differentially expressed by berry extract, sub-family B (MDR/TAP) member 1A (ABCB1A) and sub-family C (CFTR/MRP) member 4 (ABCC4) were up-regulated, whilst sub-family A (ABC1) member 8A (ABCA8A) and sub-family C (CFTR/MRP) member 9 (ABCC9) were down-regulated. ABCB1 is a transmembrane active efflux pump for xenobiotic compounds with a broad substrate specificity (Leschziner et al., 2006). The gene sequence of ABCB1 contains putative binding sites for stress-induced transcription factors, such as AP-1 and C/EBPs (Sukhai and Piquette-Miller, 2000). ABCC4 is implicated in pumping a wide variety of endogenous and xenobiotic organic anionic compounds out of the cell (Russel et al., 2008). On the other hand, ABCA8 transports certain lipophilic drugs (Tsuruoka et al., 2002) and ABCC9 is an ATP-

sensitive potassium channel (Dean et al., 2001). These findings indicated that berry extract specifically enhanced the expression of ABC transporters that remove xenobiotics from cells.

4.6.5 Extracellular structure/ cytoskeleton

Following exposure to berry extract, 14 genes involved in the regulation of extracellular/cytoskeleton were differentially expressed in C2C12 myotubes. In particular, four genes from a sub-family of leucine-rich repeats (LRRs), namely the small leucine-rich proteoglycans (SLRPs), are implicated in modulating extracellular matrix via regulation of collagen fibrillogenesis.

4.6.5.1 Small leucine-rich proteoglycans and collagen

Among the differentially expressed genes encoding LRRs, 4 of them are sub-characterised as SLRPs. These genes are asporin, fibromodulin, lumican and osteomodulin. Use of the MAS5.0 algorithm further identified a 1.7-fold down-regulation of the gene of another SLRP, namely decorin.

Various SLRPs can bind fibrillar collagens (Vogel et al., 1984, Hedbom and Heinegård, 1993, Rada et al., 1993). The interactions between SLRPs and collagens play an important role in proper collagen fibril assembly and formation of a functional extracellular matrix (ECM) (Kalamajski and Oldberg, 2010). In skeletal muscle, collagens provide external structural support for muscle cells, where they are found predominantly in three structures, namely epimysium, perimysium and endomysium (Borg and Caulfield, 1980). However, over-accumulation of collagen fibrils leads to fibrosis and is regarded unfavorable in muscle healing (Huard et al., 2002).

In addition to their role as structural proteins, SLRPs have also been identified as modulators of signalling pathways, which include those mediated by transforming growth factor-beta (TGF-beta) (Hildebrand et al., 1994, Droguett et al., 2006), insulin-like growth factor-1 (IGF-1) (Schönherr et al., 2005) and Toll-like receptor (Wu et al., 2007).

We reported here that exposure of C2C12 myotubes to berry extract negatively modulated the expression of not only several SLRPs, but also 7 genes that encode different types of collagen. These changes suggested there is some potency of berry extract in the regulation of collagen fibrillogenesis as well as in multiple signalling pathways that are involved in muscle cell growth and repair.

4.7 Effects of ethanol on gene expression of glucose transporters in C2C12 myotubes

Spolarics et al. (1994) demonstrated that acute alcohol administration (3 hours) to rats markedly attenuated basal as well as insulin-stimulated glucose use in skeletal muscles. We found, in the present study, that incubation of C2C12 myotubes with ethanol at 30-300 mM for 24 hours triggered significant down-regulation of the gene expression of the insulin-responsive GLUT4 transporter (see **Figure 39**, page 125). This finding suggested a possible mechanism by which alcohol inhibits glucose utilisation in the skeletal muscles. However, in terms of muscle or any other tissues, it is difficult to ascribe mechanisms to a particular pathology if only one or two genes are examined. Ideally, several genes or the entire gene network should be investigated. This is indeed the approach we adopted.

4.8 Global gene expression profiling of C2C12 myotubes in response to ethanol

Chronic alcoholism has been shown to cause muscle damage (Song and Rubin, 1972, Rubin et al., 1976). In the C2C12 muscle cell model, we demonstrated that 24-hour ethanol treatment induced changes in the expression of genes involved in multiple cellular functions. These include insulin signalling/ glucose metabolism, muscle metabolism and contraction, cell cycle and protein synthesis.

4.8.1 Insulin signalling/ glucose metabolism

4.8.1.1 PI3K-mediated signalling pathway

Phosphatidylinositide 3-kinase (PI3K) is a key component of the insulin signalling pathway (Tsakiridis et al., 1995). Downstream targets of the PI3K-mediated signalling pathway include the tre-2/USP6, BUB2, cdc16 domain family member 1 (TBC1D1), a Rab GTPase-activating protein which regulates GLUT4 translocation (Roach et al., 2007, Peck et al., 2009). Insulin receptor substrate-2 (IRS-2) represents one of the cytoplasmic signalling molecules that mediate signals between the transmembrane insulin receptor tyrosine kinase and its downstream effectors such as PI3K (Sun et al., 1995). In the present study, the expressions of genes encoding the beta catalytic subunit of PI3K and TBC1D1 in C2C12 cells were both down-regulated by ethanol. In contrast, a significant 30% up-regulation of the IRS-2 expression was observed in our qRT-PCR validation (although it was not altered in the microarray, possibly due to its lower detection sensitivity). Perturbations in the PI3K-mediated signalling have been reported in skeletal muscles exposed to ethanol (Qu et al., 2011, Nguyen et al., 2012). Our data confirmed that ethanol altered the PI3K-dependent insulin signalling pathway in C2C12 myotubes. The up-regulation of IRS2 might indicate a feedback mechanism in response to the defects in the downstream insulin signalling.

4.8.1.2 Insulin-like growth factor

Insulin-like growth factor 1 (IGF-1) is known to promote both cell proliferation and differentiation in muscle (Florini et al., 1991, Coolican et al., 1997). Chronic alcohol feeding to rats decreases muscle protein (i.e. a catabolic event) and IGF-1 levels in the circulation and skeletal muscle, with a concomitant increase in IGF binding protein 1 (Lang et al., 1998). Resnicoff et al. (1993) also demonstrated that alcohol markedly inhibited auto-phosphorylation of IGF-1 receptor and IGF-1-mediated cell growth in adipocytes. In contrast, increased IGF-1 mRNA expression occurred during stretch-induced muscle hypertrophy (i.e. an anabolic event) (Czerwinski et al., 1994, Yang et al., 1997). Intriguingly, we reported an up-regulation of the expression of IGF-1 in C2C12 myotubes upon ethanol treatment but a decline in IGF-binding protein 3 (IGFBP3), the latter which has been found to bind to IGF-1 and inhibit IGF-1-stimulated protein synthesis in cardiomyocytes (Ito et al., 1993). IGF-1 is primarily produced in the liver but also in target tissues acting in a paracrine/ autocrine fashion (Zapf and Froesch, 1986). The length of ethanol treatment (24 hours) in the present study was relatively short compared to chronic alcohol feeding in animal models (usually for several weeks). Thus, the changes in the levels of IGF-1 and IGFBP3 we observed in this study might reflect an initial response of skeletal muscle, as an extra-hepatic tissue, to compensate for a general perturbation in IGF-1-mediated signalling.

4.8.2 Muscle metabolism and contraction

4.8.2.1 Myosin heavy chains

Myosin heavy chains comprise the force-generating proteins in skeletal muscle. Our microarray data showed that ethanol down-regulated genes expressing two myosin heavy chain (MyHC) isoforms, myosin heavy chain IIa (MyHC-IIa) fast-twitch (MYH2) and myosin heavy chain β (MyHC-β) cardiac muscle, slow-twitch (MYH7).

However, qRT-PCR validation only confirmed the down-regulation of MyHC-β, whereas the reduction of MyHC-IIa expression was not significant. Our observations were consistent with the findings reported by Reilly et al. (2000). These authors demonstrated that in chronically ethanol-fed rats, only the MyHC-β isoform showed significant reduction at the mRNA level among all muscle MyHC types (Reilly et al., 2000). This down-regulation was observed in both type I-predominant soleus muscle and type II-predominant plantaris muscle (Reilly et al., 2000). Thus, our results appeared to resemble the *in vivo* ethanol-fed animal model in terms of changes in myosin heavy chains gene expression.

4.8.2.2 Troponin and tropomyosin

The expression of troponin I, cardiac 3 (TNNI3) and tropomyosin 1, alpha (TPM1A) were up-regulated in C2C12 myotubes exposed to ethanol as shown in the microarray. However, the change of TNNI3 did not reach statistical significance in the qRT-PCR validation. In fact, the signal intensities from the probes detecting TNNI3 were low. This is consistent with the findings that this troponin isoform is not expressed in fetal and healthy or diseased adult human skeletal muscle tissue (Bodor et al., 1995). Tropomyosin is an elongated alpha-helical coiled-coil protein that regulates muscle contraction by modulating the actin-myosin interaction (Zot and Potter, 1987). It does so by sterically hindering the myosin binding sites on actin filaments in the relaxed state. On activation triggered by an elevated Ca^{2+} concentration, tropomyosin undergoes a conformational change, exposing the binding sites on actin for myosin cross-bridge, which drives force generation and muscle contraction (Lehman et al., 2000, Gordon et al., 2001). TPM1A, the fast-twitch muscle isoform of tropomyosin, was up-regulated in response to ethanol in this study. This change might be relevant to the prevalence of muscle weakness in

chronic alcoholic misusers, which was observed in approximately half of the patients with chronic alcoholic myopathy (Urbano-Marquez et al., 1995).

4.8.2.3 Ankyrin repeat domain-containing protein 2

Ankyrin repeat domain-containing protein 2 (ANKRD2) is a stretch-responsive protein present in striated muscles (Kemp et al., 2000). Studies by Miller et al. (2003) suggested that ANKRD2 elicited stretch-induced responses by linking strain signals from titin, a myofibrillar elastic protein, to the induction of muscle-specific gene expression. Later investigations, which demonstrated the ability of ANKRD2 to translocate between the sarcomere and the nucleus in response to muscle damage, supported this notion (Kojic et al., 2004, Tsukamoto et al., 2008). Interestingly, expression of ANKRD2 is associated with slow-twitch muscles (Tsukamoto et al., 2002, Mckoy et al., 2005, Barash et al., 2007). Stretching of fast-twitch muscles up-regulated the expression of ANKRD2 and this change was in parallel with a transition towards a slow muscle phenotype (Mckoy et al., 2005). ANKRD2 was also involved in the coordination of proliferation and apoptosis during muscle differentiation (Bean et al., 2008).

In alcoholic myopathy, atrophy mainly occurs in the fast type II muscle fibres (Martin et al., 1985), whereas the slow type I fibres are relatively protected (Hanid et al., 1981, Slavin et al., 1983). The up-regulation of ANKRD2, which is mainly associated with the slow type I muscle, in C2C12 myotubes in response to ethanol we reported here led us to speculate a possible role of ANKRD2 in protecting this specific muscle type from damage by ethanol.

4.8.3 Cell cycle

Alcohol increased the expression of the proto-oncogene, c-MYC, in rat skeletal and cardiac muscles, especially in the type II fibre-predominant muscles (Paice et al., 2002, Nakahara et al., 2003). The authors postulated it was a cellular adaptation to the stress provoked by ethanol (Paice et al., 2002, Nakahara et al., 2003). c-MYC is a transcriptional activator that induces a number of positive regulators of cell cycle progression (Morrish et al., 2008), as well as genes involved in glucose metabolism, biogenesis of mitochondria, tRNAs, ribosomes and protein synthesis (Leon et al., 2009). This protein appears to function through hetero-dimerisation with the constitutively expressed bHLH-Zip protein, MAX (Ayer et al., 1993). In this study, although ethanol did not cause any changes in the expression of c-MYC, we reported a decline in the expression of MAX dimerisation protein 1 (MXD1). MXD1 competes with c-MYC for binding MAX to form a hetero-dimeric complex and antagonises the transcriptional activity of c-MYC. Thus, in our C2C12 cell model, alcohol appeared to promote the function of c-MYC by suppressing MXD1 expression.

Cyclin D1 (CCND1) is a nuclear protein required for cell cycle progression in G1 (Baldin et al., 1993), whereas NIMA (never in mitosis gene a)-related expressed kinase 8 (NEK8) is implicated in G2/M progression (Bowers and Boylan, 2004). In contrast, growth arrest-specific 1 (GAS1) plays a role in growth suppression by blocking entry to S phase (Del Sal et al., 1992). The observations that the expressions of CCND1 and NEK8 were down-regulated and that of GAS1 was up-regulated by ethanol suggested that ethanol is likely to induce cell cycle arrest. Intriguingly, c-MYC was found to suppress the expression of GAS1 (Gartel and Shchors, 2003). Taken together, the changes in the expression of genes related to

cell cycle control suggested an intricate interplay of various molecules in muscle cells to determine cell fate in response to cytotoxic agents, as represented by ethanol here.

4.8.4 Protein synthesis

Cellular mRNA translation involves the binding of the 5′-end of mRNA to the pre-initiation complex, eIF4F (Sonenberg and Dever, 2003). The activity of eIF4F depends on the formation of an active heterotrimeric complex composed of the eukaryotic initiation factors, eIF4E, eIF4A and eIF4G (Clemens, 2001). We showed that ethanol reduced the expression of eIF4E, the least abundant and presumably the most rate-limiting constituent of the active eIF4F complex in skeletal muscle (Lang et al., 2004). In addition, the MAS5.0 algorithm also indicated a 20% down-regulation of mTOR, a serine/ threonine protein kinase that phosphorylates eIF4E-binding protein and liberates eIF4E to enter the eIF4F complex (Wang et al., 2000). It has been reported that chronic alcohol consumption led to reduced muscle protein synthesis (Pacy et al., 1991) and this inhibition was associated with reduced availability of eIF4E (Lang et al., 1999). The changes we reported in this study were in agreement with the above mentioned studies, indicating that ethanol suppressed genes responsible for the initiation of protein synthesis. Interestingly, stresses that reduced eIF4E availability were also found to regulate cell cycle progression, for example, by inhibiting the synthesis of cyclin D1, which was also reduced in our study (mentioned above in the “Cell cycle” section).

4.8.5 Ethanol-treated C2C12 myotubes as an *in vitro* cell model of alcoholic myopathy

Alcoholic myopathy is one of the most common alcohol-related diseases (Martin et al., 1985, Urbano-Marquez et al., 1989, Urbano-Marquez et al., 1995). Animal

models have been developed to dissect out the pathogenic mechanisms of alcohol-induced muscle diseases (Ward et al., 1987, Preedy and Peters, 1988b, Preedy and Peters, 1988a) but the use of cell culture models is scarce (Hong-Brown et al., 2001, Qu et al., 2011). In this study, using the C2C12 muscle cell model, which expresses the alcohol dehydrogenase 1B mRNA, we evaluated the cellular consequences of ethanol treatment on muscle by measuring the changes in global gene expression with a microarray approach.

We demonstrated that exposure of differentiated C2C12 myotubes to ethanol led to the differential expression of genes that encode proteins involved in the PI3K-mediated insulin signalling pathway. These are the PI3K beta catalytic subunit, TBC1D1 and IRS2. Alterations in genes related to IGF-1 signalling and glucose metabolism were also detected. These findings are in line with impairment in muscle glucose utilisation and development of insulin insensitivity after alcohol administration (Spolarics et al., 1994, Onishi et al., 2003, Wan et al., 2005).

Furthermore, we showed that ethanol incubation suppressed the expression of eukaryotic initiation factors (eIFs) in C2C12 cells, potentially inhibiting initiation of translation and subsequently leading to reduced protein synthesis. Considering that myofibrillar proteins are responsible for force generation and its regulation during muscle contraction, we reported changes in the mRNA levels of myofibrillar proteins, including myosin heavy chains, troponin and tropomyosin. Several genes implicated in the regulation of muscle metabolism and differentiation, namely the ankyrin repeat domain-containing protein 2 (ANKRD2), carbonic anhydrase (CAR3), mitochondrial creatine kinase (CKMT) and four-and-a-half LIM protein 1 (FHL1), were also differentially expressed in response to ethanol. These findings

suggested that ethanol-treated C2C12 myotubes partly resembled skeletal muscles of alcohol-fed animals in terms of a reduction in protein synthesis and myofibrillar changes, at least at the mRNA level (Preedy and Peters, 1988a, Pacy et al., 1991, Reilly et al., 2000).

Based on the alterations in global gene expression of C2C12 myotubes we observed in response to ethanol, we propose that ethanol-treated C2C12 myotubes might serve as a potential cell model for the studying of pathogenic mechanisms of alcoholic myopathy.

4.9 Putative protective effects of berry extract against ethanol-induced cellular damage in skeletal muscle

Chronic alcoholism can lead to tissue damage not only in the skeletal muscle, but also in cardiac muscle, liver, brain, etc. (Brewer and Perrett, 1971, Rubin et al., 1976, Lee et al., 1979). Oxidative stress as a result of generation of reactive oxygen species and depletion of antioxidant enzymes, as well as acetaldehyde-protein adduct formation, during cellular ethanol metabolism, are known to contribute to the alcohol-induced cellular damage (Polavarapu et al., 1998, Meagher et al., 1999, Worrall et al., 2001). Although alcohol consumption has been shown to increase the generation of lipid peroxidation products in both humans and animals (Polavarapu et al., 1998, Meagher et al., 1999), Pignatelli et al. (2006) showed that consumption of either red or white wine in healthy subjects suppressed oxidative stress, as evident in a reduction in urinary isoprostanes, compared to participants refrained from alcoholic beverages. The authors suggested that the wine exerted its antioxidant effects in humans via its polyphenolic content (Pignatelli et al., 2006). In fact, quercetin, the most abundant flavonoid in foods, has been found to reduce ethanol-induced oxidative stress and cytotoxicity in human hepatocytes (Yao et al., 2007).

In the present study, our microarray data provided strong evidence that the flavonoid-rich berry extract is a potent inducer of the expression of genes involved in the cellular antioxidant defence system in C2C12 muscle cells. The enhancement of endogenous antioxidant defence appeared to occur by an up-regulation of anti-oxidative stress enzymes such as GSTs, NQO-1 and HMOX-1, as well as the ABC transporter, ABCB1 (also known as MDR1) (see **Figure 44**). These changes were in turn regulated by an elevated level of the antioxidant/stress-responsive transcription factors, C/EBPs and AP-1. Thus, our findings indicate that ingestion of flavonoid-rich berry extract might increase the capacity of cells to acquire an enhanced cellular antioxidant system. This potentially ameliorates the oxidative stress provoked by ethanol consumption and protects body tissue against ethanol-induced damage. However, due to time limitations within the context of this thesis, it was not possible to test the above assumption by simultaneous exposure of C2C12 muscle cells to the two substrates, i.e. berry extract and ethanol.

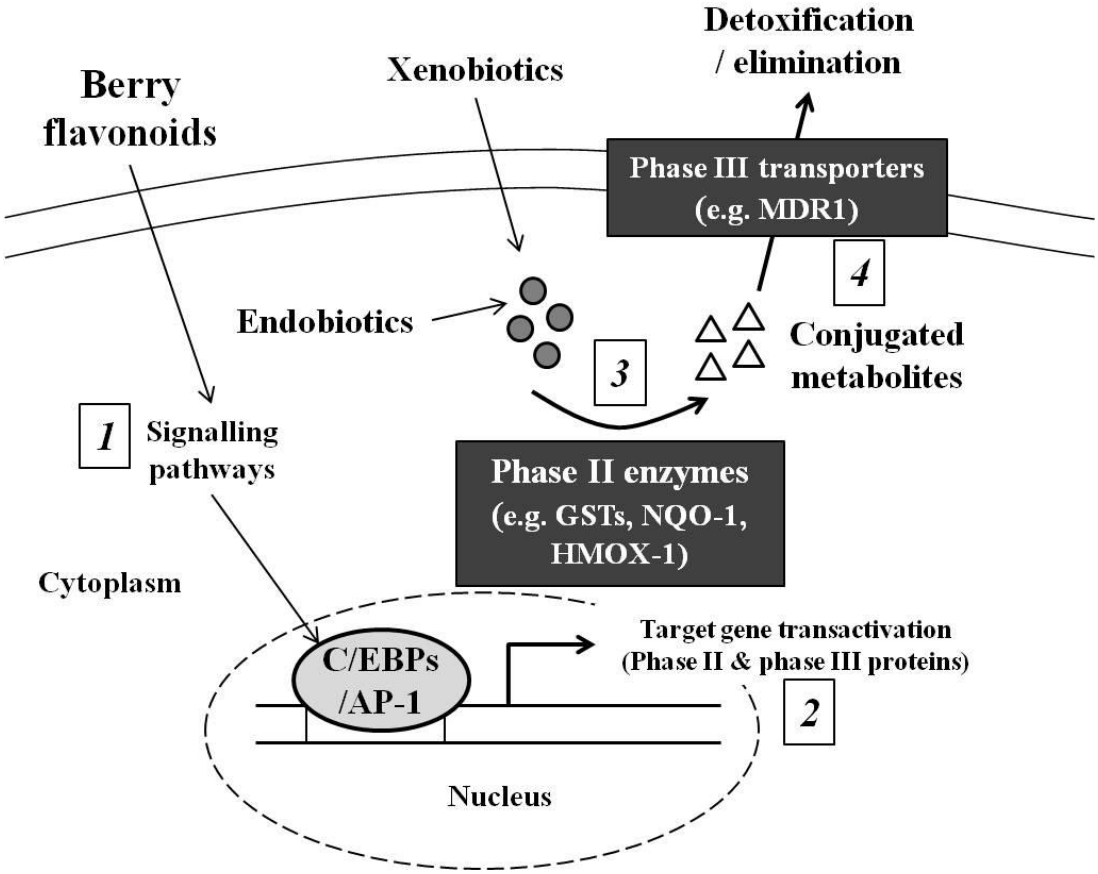

Figure 44. A schematic representation of the mechanism by which berry flavonoids enhance cellular antioxidant defence

1) Berry flavonoids activate signalling pathways that stimulate the expression of transcription factors, such as C/EBPs and AP-1; ***2***) binding of C/EBPs and/or AP-1 to specific DNA sequences transactivates the transcription of target genes including the phase II enzymes, GSTs, NQO-1 and HMOX-1, as well as the phase III transporter, MDR1; ***3***) phase II enzymes facilitate the conjugation of xenobiotics and endobiotics to glutathione; ***4***) conjugated metabolites are then excreted out of the cell via phase III transporters. Adapted from Yang et al. (2010).

5 Summary

Using the intestinal epithelial Caco-2 cell model, we demonstrated that acute exposure to flavonoid-rich berry extract inhibited glucose uptake, whereas chronic incubation down-regulated the gene expression of GLUT2 and SGLT1. The concomitant up-regulation of the miRNA, *let-7a*, following berry treatment, suggested that miRNA might be involved in the down-regulation of glucose transporter expression.

However, in C2C12 myotubes, chronic incubation with berry extract did not have any effects on either basal or insulin-stimulated glucose uptake. These findings suggested that the flavonoid-rich berry extract may be useful for regulating the rate of glucose absorption from the diet; however, it is less effective in modulating peripheral glucose transport.

Microarray studies showed that ethanol altered genes involved in multiple cellular functions in C2C12 muscle cells, including insulin signalling, muscle contraction and protein synthesis. These alterations are in line with cellular changes that occur in alcoholic myopathy. Thus, we proposed that ethanol-treated C2C12 myotubes might act as a cell model for alcoholic myopathy.

Microarray studies also revealed that flavonoid-rich berry extract enhanced the endogenous antioxidant defence system in C2C12 myotubes. This occurred via up-regulation of oxidative stress defence enzymes such as GSTs, NQO-1 and HMOX-1, which were in turn regulated by the antioxidant-responsive transcription factors, C/EBPs and AP-1. Thus, we postulated that berry extract might be beneficial to protect against tissue damage induced by ethanol, which is known to increase cellular oxidative stress.

6 Future investigations

Based on the studies performed and presented in this thesis, a few further investigations are proposed to substantiate our initial findings and to take the work into a forward trajectory, as discussed in the following section. Rather than providing lengthy text on all areas, a more detailed discourse is provided for the miRNA transfection studies and alcohol studies.

6.1 Identification of chemical constituents of berry extract

The OptiBerry® berry extract used in the present study was a commercialised flavonoid-rich extract of wild blueberry, strawberry, cranberry, wild bilberry, elderberry and raspberry. The analysis report given by the manufacturer provided limited information on its phytochemical components- it only specifies the approximate content of a few anthocyanidins, namely cyanidin, delphinidin, malvidin and petunidin.

However, in the literature, a much wider range of polyphenolic components has been identified and characterised in the abovementioned berries. These include flavonols, e.g. quercetin, myricetin, kaempferol; and phenolic acids, e.g. ellagic acid, *p*-coumaric acid and caffeic acid (Zheng et al., 2007, Latti et al., 2010, Gavrilova et al., 2011, Vrhovsek et al., 2012). Since these polyphenolic compounds also have modulatory effects on glucose uptake, it would be useful to perform an extensive phytochemical profiling of the commercial OptiBerry® berry extract. This can be achieved by carrying out high performance liquid chromatography (HPLC) or nuclear magnetic resonance spectroscopy (NMR) analyses.

6.2 Regulation of GLUT2 expression by *let-7a* miRNA

In our Caco-2 studies, we reported an up-regulation of the miRNA, *let*-7a, along with a down-regulation of GLUT2 and SGLT1 expression in response to chronic treatment with berry extract (16 hours). Bioinformatics analysis showed that the 3'-untranslated regulatory region of GLUT2 mRNA is a predicted target of *let-7a*. This suggested a possible role for *let-7a* as a negative post-transcriptional regulator of GLUT2 expression. Further studies using miRNA transfection techniques will be required to test this hypothesis.

Briefly, expression of GLUT2 mRNA will be measured in two experimental conditions with or without berry extract, as described below:

1) Transfection of *let-7a* miRNA precursor;
2) Transfection of anti-*let-7a* oligonucleotide that blocks the binding activity of *let-7a*.

In addition, to determine whether *let-7a* regulates GLUT2 expression through the predicted *let-7a* binding site on the 3'-UTR of GLUT2, luciferase activity will also be measured in the following conditions, with or without berry extract:

1) Co-transfection of *let-7a* precursor and the predicted *let-7a* binding site on the 3'-UTR of GLUT2, cloned upstream of a luciferase reporter plasmid;
2) Co-transfection of *let-7a* precursor and a mutated *let-7a* binding site on the 3'-UTR of GLUT2, cloned upstream of a luciferase reporter plasmid.

6.3 Involvement of signalling pathways in the acute regulation of glucose uptake in muscle by berry extract

In C2C12 cells, berry extract did not exert any effects on glucose transport at the transcriptional level after chronic pre-incubation (24 hours). However, it is worth

noting that glucose transport in muscle is also under intricate and rapid regulation at the translational as well as post-translational levels. This is evident in the observations that insulin and muscle contraction can stimulate glucose uptake within minutes (Lund et al., 1995, Sun et al., 2010). Therefore, future work will include investigations to identify whether berry extract exert acute effects on glucose uptake and to elucidate the putative involvement of signalling pathways in the acute regulation of glucose uptake in muscle. Translocation of glucose transporters in response to berry extract treatment should also be studied.

6.4 Validation of ethanol-induced cellular changes in C2C12 muscle cells

We characterised the changes in global gene expression in C2C12 myotubes in response to ethanol. Alterations in the expression of genes involved in insulin signalling, muscle contraction and protein synthesis were observed. These changes were generally in agreement with *in vivo* findings reported in alcoholic myopathy or its corresponding animal models. However, further investigations are needed to verify the cellular changes at the protein and functional levels. These include the measurements of specific myosin heavy chain proteins and the activities of kinases along the PI3K-mediated insulin signalling cascade.

6.5 Putative protective effects of berry extract against ethanol-induced cellular damage in muscle

In the microarray studies, we reported the changes in global gene expression in C2C12 myotubes in response to berry extract or ethanol. Our results presented preliminary evidence that the flavonoid-rich berry extract increased the capacity of muscle cells to acquire an enhanced endogenous antioxidant defence system. This occurred in part by elevating the levels of antioxidant-responsive transcription

factors which in turn was associated with an increase in the expression of genes encoding oxidative stress defence enzymes, such as glutathione-s-transferase and heme oxygenase 1. Since oxidative stress has been shown to contribute to the perturbations in cellular integrity as a consequence of ethanol ingestion (Mantle and Preedy, 1999, Wu and Cederbaum, 2003), berry extract might serve as a dietary strategy to ameliorate ethanol-induced cellular damage. Due to time limitations, we were not able to test this aforementioned hypothesis directly. It is proposed that future work to investigate the cellular consequences of simultaneous incubation with berry extract and ethanol in C2C12 muscle cells should be undertaken for this purpose. Parameters to be measured will include cellular antioxidant status (changes in the level of lipid peroxidation products such as malondialdehyde, and changes in glutathione content and glutathione peroxidase activity) and formation of aldehyde-protein adducts.

For example, malondialdehyde-acetaldehyde adducts are produced when there is simultaneous oxidative stress and acetaldehyde formation (Niemelä et al., 2002). Hydroxyethyl radical adducts are produced as a consequence of free radical formation, albeit as a result of ethanol metabolism, i.e. hydroxyethyl radicals are only produced produced as a result of ethanol metabolism (Patel et al., 2005). Immunohistochemistry will determine their locations, while ELISA the relative abundance of the aforementioned protein adducts.

However, the studies prosposed above do not ascribe changes to either ethanol or acetaldehyde. In order to identify which analyte contributes to specific cellular changes, experiments can be carried out with the simultaneous addition of ethanol and 4-methylpyrazole. 4-methylpyrazole is an inhibitor of alcohol dehydrogenase

which blocks ethanol metabolism and diminishes the level of acetaldehyde. Thus, observations may potentially be ascribed to ethanol *per se* rather than acetaldehyde. The addition of cyanamide, an inhibitor of aldehyde dehydrogenase, will increase acetaldehyde concentration. Thus, the use of cyanamide will show if raising endogenous acetaldehyde will exacerbate any effects (Niemelä et al., 2002). The use of acetaldehyde alone will also substantiate these findings, though acetaldehyde is extremely volatile, so careful monitoring may be required during these experimental protocols.

7 References

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8 Appendix

Table 26. List of up-regulated genes in C2C12 myotubes upon treatment with 0.125% (w/v) berry extract

Affymetrix ID	Gene symbol	Gene name	Signal log ratio	Fold change
1456718_at	*TMEM56*	Transmembrane protein 56	6.0	64.00
1450826_a_at	*SAA3*	Serum amyloid A 3	5.0	32.00
1423436_at	*GSTA3*	Glutathione S-transferase, alpha 3	4.7	25.99
1419209_at	*CXCL1*	Chemokine (C-X-C motif) ligand 1	4.4	21.11
1419728_at	*CXCL5*	Chemokine (C-X-C motif) ligand 5	4.3	19.70
1421040_a_at	*GSTA2*	Glutathione S-transferase, alpha 2 (Yc2)	3.7	13.00
1420380_at	*CCL2*	Chemokine (C-C motif) ligand 2	3.6	12.13
1451054_at	*ORM1*	Orosomucoid 1	3.4	10.56
1449824_at	*PRG4*	Proteoglycan 4 (megakaryocyte stimulating factor, articular superficial zone protein)	3.2	9.19
1456225_x_at	*TRIB3*	Tribbles homolog 3 (Drosophila)	3.0	8.00
1427747_a_at	*LCN2*	Lipocalin 2	2.9	7.46
1443536_at	*SLC7A11*	Solute carrier family 7 (cationic amino acid transporter, y+ system), member 11	2.9	7.46
1421001_a_at	*CAR6*	Carbonic anhydrase 6	2.8	6.96
1449195_s_at	*CXCL16*	Chemokine (C-X-C motif) ligand 16	2.5	5.66
1421041_s_at	*GSTA1*	Glutathione S-transferase, alpha 1 (Ya)	2.5	5.66
1418752_at	*ALDH3A1*	Aldehyde dehydrogenase family 3, subfamily A1	2.4	5.28
1426243_at	*CTH*	Cystathionase (cystathionine gamma-lyase)	2.3	4.92
1421471_at	*NPY1R*	Neuropeptide Y receptor Y1	2.3	4.92
1417130_s_at	*ANGPTL4*	Angiopoietin-like 4	2.2	4.59
1421228_at	*CCL7*	Chemokine (C-C motif) ligand 7	2.2	4.59
1450882_s_at	*GPR137B*	G protein-coupled receptor 137B	2.1	4.29
1450075_at	*POLH*	Polymerase (DNA directed), eta (RAD 30 related)	2.1	4.29
1438488_at	*ESD*	Esterase D/formylglutathione hydrolase	2.0	4.00
1458245_at	*SUSD1*	Sushi domain containing 1	1.9	3.73
1443870_at	*ABCC4*	ATP-binding cassette, sub-family C (CFTR/MRP), member 4	1.8	3.48
1423233_at	*CEBPD*	CCAAT/enhancer binding protein (C/EBP), delta	1.8	3.48
1416368_at	*GSTA4*	Glutathione S-transferase, alpha 4	1.8	3.48
1448239_at	*HMOX1*	Heme oxygenase (decycling) 1	1.8	3.48
1448377_at	*SLPI*	Secretory leukocyte peptidase inhibitor	1.8	3.48
1451006_at	*XDH*	Xanthine dehydrogenase	1.8	3.48
1436990_s_at	*CHCHD10*	Coiled-coil-helix-coiled-coil-helix domain containing 10	1.7	3.25
1455660_at	*CSF2RB*	Colony stimulating factor 2 receptor, beta, low-affinity (granulocyte-macrophage)	1.7	3.25
1448469_at	*NID1*	Nidogen 1	1.7	3.25

To be cont'd

Affymetrix ID	Gene symbol	Gene name	Signal log ratio	Fold change
1418666_at	*PTX3*	Pentraxin related gene	1.7	3.25
1441315_s_at	*SLC19A2*	Solute carrier family 19 (thiamine transporter), member 2	1.7	3.25
1419456_at	*DCXR*	Dicarbonyl L-xylulose reductase	1.6	3.03
1418627_at	*GCLM*	Glutamate-cysteine ligase, modifier subunit	1.6	3.03
1423627_at	*NQO1*	NAD(P)H dehydrogenase, quinone 1	1.6	3.03
1420664_s_at	*PROCR*	Protein C receptor, endothelial	1.6	3.03
1417263_at	*PTGS2*	Prostaglandin-endoperoxide synthase 2	1.6	3.03
1424938_at	*STEAP1*	Six transmembrane epithelial antigen of the prostate 1	1.6	3.03
1421058_at	*ADH7*	Alcohol dehydrogenase 7 (class IV), mu or sigma polypeptide	1.5	2.83
1454822_x_at	*APCDD1*	Adenomatosis polyposis coli down-regulated 1	1.5	2.83
1434866_x_at	*CPT1A*	Carnitine palmitoyltransferase 1a, liver	1.5	2.83
1419029_at	*ERO1L*	ERO1-like (S. Cerevisiae)	1.5	2.83
1435748_at	*GDA*	Guanine deaminase	1.5	2.83
1418248_at	*GLA*	Galactosidase, alpha	1.5	2.83
1423706_a_at	*PGD*	Phosphogluconate dehydrogenase	1.5	2.83
1419758_at	*ABCB1A*	ATP-binding cassette, sub-family B (MDR/TAP), member 1A	1.4	2.64
1427844_a_at	*CEBPB*	CCAAT/enhancer binding protein (C/EBP), beta	1.4	2.64
1427302_at	*ENPP3*	Ectonucleotide pyrophosphatase/phosphodiesterase 3	1.4	2.64
1416416_x_at	*GSTM1*	Glutathione S-transferase, mu 1	1.4	2.64
1421034_a_at	*IL4RA*	Interleukin 4 receptor, alpha	1.4	2.64
1418400_at	*LARP6*	La ribonucleoprotein domain family, member 6	1.4	2.64
1434322_at	*MICALL2*	MICAL-like 2	1.4	2.64
1453924_a_at	*PTGFR*	Prostaglandin F receptor	1.4	2.64
1455936_a_at	*RBPMS*	RNA binding protein gene with multiple splicing	1.4	2.64
1450387_s_at	*AK4*	Adenylate kinase 4	1.3	2.46
1452365_at	*CSGALNACT1*	Chondroitin sulfate N-acetylgalactosaminyltransferase 1	1.3	2.46
1423488_at	*MMD*	Monocyte to macrophage differentiation-associated	1.3	2.46
1437250_at	*MREG*	Melanoregulin	1.3	2.46
1456653_a_at	*MTHFD1L*	Methylenetetrahydrofolate dehydrogenase (NADP+ dependent) 1-like	1.3	2.46
1450976_at	*NDRG1*	N-myc downstream regulated gene 1	1.3	2.46
1449005_at	*SLC16A3*	Solute carrier family 16 (monocarboxylic acid transporters), member 3	1.3	2.46
1455899_x_at	*SOCS3*	Suppressor of cytokine signaling 3	1.3	2.46
1415997_at	*TXNIP*	Thioredoxin interacting protein	1.3	2.46
1449363_at	*ATF3*	Activating transcription factor 3	1.2	2.30
1451386_at	*BLVRB*	Biliverdin reductase B (flavin reductase (NADPH))	1.2	2.30
1417574_at	*CXCL12*	Chemokine (C-X-C motif) ligand 12	1.2	2.30

To be cont'd

Affymetrix ID	Gene symbol	Gene name	Signal log ratio	Fold change
1422438_at	*EPHX1*	Epoxide hydrolase 1, microsomal	1.2	2.30
1420654_a_at	*GBE1*	Glucan (1,4-alpha-), branching enzyme 1	1.2	2.30
1418949_at	*GDF15*	Growth differentiation factor 15	1.2	2.30
1421973_at	*GFRA1*	Glial cell line derived neurotrophic factor family receptor alpha 1	1.2	2.30
1415897_a_at	*MGST1*	Microsomal glutathione S-transferase 1	1.2	2.30
1452592_at	*MGST2*	Microsomal glutathione S-transferase 2	1.2	2.30
1452620_at	*PCK2*	Phosphoenolpyruvate carboxykinase 2 (mitochondrial)	1.2	2.30
1434773_a_at	*SLC2A1*	Solute carrier family 2 (facilitated glucose transporter), member 1	1.2	2.30
1450409_a_at	*SLC48A1*	Solute carrier family 48 (heme transporter), member 1	1.2	2.30
1460243_at	*SPTLC2*	Serine palmitoyltransferase, long chain base subunit 2	1.2	2.30
1417172_at	*UBE2L6*	Ubiquitin-conjugating enzyme E2L 6	1.2	2.30
1438165_x_at	*VAT1*	Vesicle amine transport protein 1 homolog (T californica)	1.2	2.30
1454617_at	*ARRDC3*	Arrestin domain containing 3	1.1	2.14
1426334_a_at	*BCL2L11*	BCL2-like 11 (apoptosis facilitator)	1.1	2.14
1451382_at	*CHAC1*	Chac, cation transport regulator 1	1.1	2.14
1426893_at	*FAM102A*	Family with sequence similarity 102, member A	1.1	2.14
1438953_at	*FIGF*	C-fos induced growth factor	1.1	2.14
1437247_at	*FOSL2*	Fos-like antigen 2	1.1	2.14
1418364_a_at	*FTL1*	Ferritin light chain 1	1.1	2.14
1448354_at	*G6PDX*	Glucose-6-phosphate dehydrogenase X-linked	1.1	2.14
1438009_at	*HIST1H2AP*	Histone cluster 1, h2ap	1.1	2.14
1419647_a_at	*IER3*	Immediate early response 3	1.1	2.14
1450161_at	*IKBKG*	Inhibitor of kappab kinase gamma	1.1	2.14
1420961_a_at	*IVNS1ABP*	Influenza virus NS1A binding protein	1.1	2.14
1453304_s_at	*LY6E*	Lymphocyte antigen 6 complex, locus E	1.1	2.14
1429274_at	*LYPD6B*	LY6/PLAUR domain containing 6B	1.1	2.14
1418936_at	*MAFF*	V-maf musculoaponeurotic fibrosarcoma oncogene family, protein F (avian)	1.1	2.14
1417234_at	*MMP11*	Matrix metallopeptidase 11	1.1	2.14
1438157_s_at	*NFKBIA*	Nuclear factor of kappa light polypeptide gene enhancer in B cells inhibitor, alpha	1.1	2.14
1440263_at	*NRP*	Neural regeneration protein	1.1	2.14
1427977_x_at	*OOG1*	Oogenesin 1	1.1	2.14
1439148_a_at	*PFKL*	Phosphofructokinase, liver, B-type	1.1	2.14
1448318_at	*PLIN2*	Perilipin 2	1.1	2.14
1452226_at	*RCC2*	Regulator of chromosome condensation 2	1.1	2.14
1426792_s_at	*RUSC2*	RUN and SH3 domain containing 2	1.1	2.14
1415802_at	*SLC16A1*	Solute carrier family 16 (monocarboxylic acid transporters), member 1	1.1	2.14
1417639_at	*SLC22A4*	Solute carrier family 22 (organic cation transporter), member 4	1.1	2.14
1420641_a_at	*SQRDL*	Sulfide quinone reductase-like (yeast)	1.1	2.14

To be cont'd

Affymetrix ID	Gene symbol	Gene name	Signal log ratio	Fold change
1416077_at	*ADM*	Adrenomedullin	1.0	2.00
1437333_x_at	*ALDH18A1*	Aldehyde dehydrogenase 18 family, member A1	1.0	2.00
1422573_at	*AMPD3*	Adenosine monophosphate deaminase 3	1.0	2.00
1433966_x_at	*ASNS*	Asparagine synthetase	1.0	2.00
1425814_a_at	*CALCRL*	Calcitonin receptor-like	1.0	2.00
1448205_at	*CCNB1*	Cyclin B1	1.0	2.00
1418982_at	*CEBPA*	CCAAT/enhancer binding protein (C/EBP), alpha	1.0	2.00
1434976_x_at	*EIF4EBP1*	Eukaryotic translation initiation factor 4E binding protein 1	1.0	2.00
1427578_a_at	*EIF6*	Eukaryotic translation initiation factor 6	1.0	2.00
1426655_a_at	*FAM63A*	Family with sequence similarity 63, member A	1.0	2.00
1419486_at	*FOXC1*	Forkhead box C1	1.0	2.00
1438169_a_at	*FRMD4B*	FERM domain containing 4B	1.0	2.00
1419080_at	*GDNF*	Glial cell line derived neurotrophic factor	1.0	2.00
1448183_a_at	*HIF1A*	Hypoxia inducible factor 1, alpha subunit	1.0	2.00
1421992_a_at	*IGFBP4*	Insulin-like growth factor binding protein 4	1.0	2.00
1428948_at	*KCNMA1*	Potassium large conductance calcium-activated channel, subfamily M, alpha member 1	1.0	2.00
1438908_at	*MAP3K12*	Mitogen-activated protein kinase kinase kinase 12	1.0	2.00
1428942_at	*MT2*	Metallothionein 2	1.0	2.00
1449043_at	*NAGA*	N-acetyl galactosaminidase, alpha	1.0	2.00
1437621_x_at	*PHGDH*	3-phosphoglycerate dehydrogenase	1.0	2.00
1456003_a_at	*SLC1A4*	Solute carrier family 1 (glutamate/neutral amino acid transporter), member 4	1.0	2.00
1425364_a_at	*SLC3A2*	Solute carrier family 3 (activators of dibasic and neutral amino acid transport), member 2	1.0	2.00
1417695_a_at	*SOAT1*	Sterol O-acyltransferase 1	1.0	2.00
1448123_s_at	*TGFBI*	Transforming growth factor, beta induced	1.0	2.00
1422587_at	*TMEM45A*	Transmembrane protein 45a	1.0	2.00
1426538_a_at	*TRP53*	Transformation related protein 53	1.0	2.00
1416926_at	*TRP53INP1*	Transformation related protein 53 inducible nuclear protein 1	1.0	2.00
1426472_at	*ZFP52*	Zinc finger protein 52	1.0	2.00
1447775_x_at	*ZFP622*	Zinc finger protein 622	1.0	2.00

Table 27. List of down-regulated genes in C2C12 myotubes upon treatment with 0.125% (w/v) berry extract

Affymetrix ID	Gene symbol	Gene name	Signal log ratio	Fold change
1437433_at	*B3GALT2*	UDP-Gal:betaglcnac beta 1,3-galactosyltransferase, polypeptide 2	-5.6	0.02
1424963_at	*RP1*	Retinitis pigmentosa 1 (human)	-4.9	0.03
1437752_at	*LIN28A*	Lin-28 homolog A (C. Elegans)	-4.6	0.04
1425298_a_at	*NAIP1*	NLR family, apoptosis inhibitory protein 1	-3.7	0.08
1456629_at	*KANK3*	KN motif and ankyrin repeat domains 3	-3.4	0.09
1455086_at	*UCHL5*	Ubiquitin carboxyl-terminal esterase L5	-3.4	0.09
1422051_a_at	*GABBR1*	Gamma-aminobutyric acid (GABA) B receptor, 1	-3.3	0.10
1418471_at	*PGF*	Placental growth factor	-3.3	0.10
1437602_at	*FAM71B*	Family with sequence similarity 71, member B	-3.1	0.12
1438700_at	*FNBP4*	Formin binding protein 4	-3.1	0.12
1457922_at	*UNG*	Uracil DNA glycosylase	-3.0	0.13
1418745_at	*OMD*	Osteomodulin	-2.9	0.13
1426851_a_at	*NOV*	Nephroblastoma overexpressed gene	-2.8	0.14
1448254_at	*PTN*	Pleiotrophin	-2.8	0.14
1452195_s_at	*SFI1*	Sfi1 homolog, spindle assembly associated (yeast)	-2.8	0.14
1448421_s_at	*ASPN*	Asporin	-2.6	0.16
1436932_at	*GRHL3*	Grainyhead-like 3 (Drosophila)	-2.6	0.16
1421589_at	*KRT31*	Keratin 31	-2.6	0.16
1425712_at	*BC025446*	Cdna sequence BC025446	-2.5	0.18
1430370_at	*EPRS*	Glutamyl-prolyl-trna synthetase	-2.5	0.18
1456344_at	*TNC*	Tenascin C	-2.5	0.18
1450004_at	*TSLP*	Thymic stromal lymphopoietin	-2.5	0.18
1427496_at	*CEP152*	Centrosomal protein 152	-2.4	0.19
1456145_at	*DLEU2*	Deleted in lymphocytic leukemia, 2	-2.4	0.19
1418152_at	*HMGN5*	High-mobility group nucleosome binding domain 5	-2.4	0.19
1452465_at	*MYH1*	Myosin, heavy polypeptide 1, skeletal muscle, adult	-2.3	0.20
1450961_a_at	*TCEAL3*	Transcription elongation factor A (SII)-like 3	-2.3	0.20
1426142_a_at	*TRDN*	Triadin	-2.3	0.20
1441161_at	*B230216G23RIK*	RIKEN cdna B230216G23 gene	-2.2	0.22
1423607_at	*LUM*	Lumican	-2.2	0.22
1425087_at	*2310003F16RIK*	RIKEN cdna 2310003F16 gene	-2.1	0.23
1428083_at	*NEAT1*	Nuclear paraspeckle assembly transcript 1 (non-protein coding)	-2.1	0.23
1455087_at	*D7ERTD715E*	DNA segment, Chr 7, ERATO Doi 715, expressed	-2.0	0.25
1436222_at	*GAS5*	Growth arrest specific 5	-2.0	0.25
1427417_at	*SCML4*	Sex comb on midleg-like 4 (Drosophila)	-2.0	0.25
1426633_s_at	*KCTD14*	Potassium channel tetramerisation domain containing 14	-1.9	0.27
1438080_at	*MRPL11*	Mitochondrial ribosomal protein L11	-1.9	0.27

To be cont'd

Affymetrix ID	Gene symbol	Gene name	Signal log ratio	Fold change
1427026_at	*MYH4*	Myosin, heavy polypeptide 4, skeletal muscle	-1.9	0.27
1438193_at	*NRXN3*	Neurexin III	-1.9	0.27
1429504_at	*RNPC3*	RNA-binding region (RNP1, RRM) containing 3	-1.9	0.27
1436429_at	*ZFP606*	Zinc finger protein 606	-1.9	0.27
1435752_s_at	*ABCC9*	ATP-binding cassette, sub-family C (CFTR/MRP), member 9	-1.8	0.29
1424608_a_at	*BZW2*	Basic leucine zipper and W2 domains 2	-1.8	0.29
1435760_at	*CSTA*	Cystatin A	-1.8	0.29
1422850_at	*PABPN1*	Poly(A) binding protein, nuclear 1	-1.8	0.29
1426863_at	*RBMX*	RNA binding motif protein, X chromosome	-1.8	0.29
1438501_at	*RPS17*	Ribosomal protein S17	-1.8	0.29
1435744_at	*6720401G13RIK*	RIKEN cdna 6720401G13 gene	-1.7	0.31
1431754_at	*CCDC173*	Coiled-coil domain containing 173	-1.7	0.31
1437512_x_at	*EBNA1BP2*	EBNA1 binding protein 2	-1.7	0.31
1455872_at	*FAM167A*	Family with sequence similarity 167, member A	-1.7	0.31
1429637_at	*FAM198B*	Family with sequence similarity 198, member B	-1.7	0.31
1437685_x_at	*FMOD*	Fibromodulin	-1.7	0.31
1416630_at	*ID3*	Inhibitor of DNA binding 3	-1.7	0.31
1418697_at	*INMT*	Indolethylamine N-methyltransferase	-1.7	0.31
1441317_x_at	*JAKMIP1*	Janus kinase and microtubule interacting protein 1	-1.7	0.31
1436218_at	*LGR6*	Leucine-rich repeat-containing G protein-coupled receptor 6	-1.7	0.31
1436540_at	*MIRLET7D*	Microrna let7d	-1.7	0.31
1442769_at	*MYBPC1*	Myosin binding protein C, slow-type	-1.7	0.31
1427470_s_at	*NAPB*	N-ethylmaleimide sensitive fusion protein attachment protein beta	-1.7	0.31
1444480_at	*PRKAG3*	Protein kinase, AMP-activated, gamma 3 non-catatlytic subunit	-1.7	0.31
1449500_at	*SERPINB7*	Serine (or cysteine) peptidase inhibitor, clade B, member 7	-1.7	0.31
1417860_a_at	*SPON2*	Spondin 2, extracellular matrix protein	-1.7	0.31
1458087_at	*STAC3*	SH3 and cysteine rich domain 3	-1.7	0.31
1422231_a_at	*TNFRSF25*	Tumor necrosis factor receptor superfamily, member 25	-1.7	0.31
1434493_at	*1810022K09RIK*	RIKEN cdna 1810022K09 gene	-1.6	0.33
1425274_at	*ASPH*	Aspartate-beta-hydroxylase	-1.6	0.33
1418440_at	*COL8A1*	Collagen, type VIII, alpha 1	-1.6	0.33
1417403_at	*ELOVL6*	ELOVL family member 6, elongation of long chain fatty acids (yeast)	-1.6	0.33
1437184_at	*GUF1*	GUF1 gtpase homolog (S. Cerevisiae)	-1.6	0.33
1439251_at	*IDUA*	Alpha-L-iduronidase	-1.6	0.33
1454966_at	*ITGA8*	Integrin alpha 8	-1.6	0.33
1439847_s_at	*KLF12*	Kruppel-like factor 12	-1.6	0.33
1417686_at	*LGALS12*	Lectin, galactose binding, soluble 12	-1.6	0.33

To be cont'd

Affymetrix ID	Gene symbol	Gene name	Signal log ratio	Fold change
1434709_at	*NRCAM*	Neuron-glia-CAM-related cell adhesion molecule	-1.6	0.33
1435341_at	*PPIG*	Peptidyl-prolyl isomerase G (cyclophilin G)	-1.6	0.33
1455195_at	*RPS24*	Ribosomal protein S24	-1.6	0.33
1423280_at	*STMN2*	Stathmin-like 2	-1.6	0.33
1439937_at	*TM9SF1*	Transmembrane 9 superfamily member 1	-1.6	0.33
1436294_at	*ANKRD29*	Ankyrin repeat domain 29	-1.5	0.35
1452732_at	*ASPRV1*	Aspartic peptidase, retroviral-like 1	-1.5	0.35
1428397_at	*B3GALT5*	UDP-Gal:betaglcnac beta 1,3-galactosyltransferase, polypeptide 5	-1.5	0.35
1455771_at	*BZRAP1*	Benzodiazepine receptor associated protein 1	-1.5	0.35
1430584_s_at	*CAR3*	Carbonic anhydrase 3	-1.5	0.35
1437025_at	*CD28*	CD28 antigen	-1.5	0.35
1419477_at	*CLEC2D*	C-type lectin domain family 2, member d	-1.5	0.35
1456887_at	*CMKLR1*	Chemokine-like receptor 1	-1.5	0.35
1440911_at	*COL23A1*	Collagen, type XXIII, alpha 1	-1.5	0.35
1419959_s_at	*CPHX*	Cytoplasmic polyadenylated homeobox	-1.5	0.35
1435343_at	*DOCK10*	Dedicator of cytokinesis 10	-1.5	0.35
1418511_at	*DPT*	Dermatopontin	-1.5	0.35
1434202_a_at	*FAM107A*	Family with sequence similarity 107, member A	-1.5	0.35
1456784_at	*GM14137*	Predicted gene 14137	-1.5	0.35
1424252_at	*HNRPDL*	Heterogeneous nuclear ribonucleoprotein D-like	-1.5	0.35
1455180_at	*MYZAP*	Myocardial zonula adherens protein	-1.5	0.35
1421566_at	*PET2*	Plasmacytoma expressed transcript 2	-1.5	0.35
1419820_at	*PKHD1*	Polycystic kidney and hepatic disease 1	-1.5	0.35
1455696_a_at	*PRPF4B*	PRP4 pre-mrna processing factor 4 homolog B (yeast)	-1.5	0.35
1443921_at	*RANBP3L*	RAN binding protein 3-like	-1.5	0.35
1425523_at	*RBM25*	RNA binding motif protein 25	-1.5	0.35
1448754_at	*RBP1*	Retinol binding protein 1, cellular	-1.5	0.35
1450734_at	*SEC16B*	SEC16 homolog B (S. Cerevisiae)	-1.5	0.35
1417305_at	*SPEG*	SPEG complex locus	-1.5	0.35
1436602_x_at	*CACNA1B*	Calcium channel, voltage-dependent, N type, alpha 1B subunit	-1.4	0.38
1424131_at	*COL6A3*	Collagen, type VI, alpha 3	-1.4	0.38
1459885_s_at	*COX7C*	Cytochrome c oxidase, subunit viic	-1.4	0.38
1417307_at	*DMD*	Dystrophin, muscular dystrophy	-1.4	0.38
1443745_s_at	*DMP1*	Dentin matrix protein 1	-1.4	0.38
1427298_at	*DNM3OS*	Dynamin 3, opposite strand	-1.4	0.38
1436434_at	*E2F2*	E2F transcription factor 2	-1.4	0.38
1425589_at	*HSD17B13*	Hydroxysteroid (17-beta) dehydrogenase 13	-1.4	0.38
1423804_a_at	*IDI1*	Isopentenyl-diphosphate delta isomerase	-1.4	0.38
1436037_at	*ITGA4*	Integrin alpha 4	-1.4	0.38
1430447_a_at	*LAIR1*	Leukocyte-associated Ig-like receptor 1	-1.4	0.38

To be cont'd

Affymetrix ID	Gene symbol	Gene name	Signal log ratio	Fold change
1431071_at	*MTX3*	Metaxin 3	-1.4	0.38
1435934_at	*NDUFAB1*	NADH dehydrogenase (ubiquinone) 1, alpha/beta subcomplex, 1	-1.4	0.38
1426003_at	*NTRK3*	Neurotrophic tyrosine kinase, receptor, type 3	-1.4	0.38
1418252_at	*PADI2*	Peptidyl arginine deiminase, type II	-1.4	0.38
1423325_at	*PNN*	Pinin	-1.4	0.38
1429052_at	*PTPRD*	Protein tyrosine phosphatase, receptor type, D	-1.4	0.38
1417214_at	*RAB27B*	RAB27b, member RAS oncogene family	-1.4	0.38
1435080_x_at	*SFRS18*	Serine/arginine-rich splicing factor 18	-1.4	0.38
1422644_at	*SH3BGR*	SH3-binding domain glutamic acid-rich protein	-1.4	0.38
1436698_x_at	*TMEM204*	Transmembrane protein 204	-1.4	0.38
1447100_s_at	*5730508B09RIK*	RIKEN cdna 5730508B09 gene	-1.3	0.41
1433801_at	*9930012K11RIK*	RIKEN cdna 9930012K11 gene	-1.3	0.41
1441054_at	*APOL8*	Apolipoprotein L 8	-1.3	0.41
1417704_a_at	*ARHGAP6*	Rho gtpase activating protein 6	-1.3	0.41
1454149_a_at	*CCNL2*	Cyclin L2	-1.3	0.41
1438540_at	*COL25A1*	Collagen, type XXV, alpha 1	-1.3	0.41
1425476_at	*COL4A5*	Collagen, type IV, alpha 5	-1.3	0.41
1425698_a_at	*CREBZF*	CREB/ATF bzip transcription factor	-1.3	0.41
1422812_at	*CXCR6*	Chemokine (C-X-C motif) receptor 6	-1.3	0.41
1437256_at	*DCUN1D5*	DCN1, defective in cullin neddylation 1, domain containing 5 (S. Cerevisiae)	-1.3	0.41
1429299_at	*DDAH1*	Dimethylarginine dimethylaminohydrolase 1	-1.3	0.41
1428960_at	*ENKUR*	Enkurin, TRPC channel interacting protein	-1.3	0.41
1438718_at	*FGF9*	Fibroblast growth factor 9	-1.3	0.41
1435459_at	*FMO2*	Flavin containing monooxygenase 2	-1.3	0.41
1451776_s_at	*HOPX*	HOP homeobox	-1.3	0.41
1439885_at	*HOXC5*	Homeobox C5	-1.3	0.41
1449872_at	*HSPB3*	Heat shock protein 3	-1.3	0.41
1440235_at	*ITGA10*	Integrin, alpha 10	-1.3	0.41
1445841_at	*LRRC39*	Leucine rich repeat containing 39	-1.3	0.41
1424802_a_at	*LUC7L3*	LUC7-like 3 (S. Cerevisiae)	-1.3	0.41
1433758_at	*NISCH*	Nischarin	-1.3	0.41
1419392_at	*PCLO*	Piccolo (presynaptic cytomatrix protein)	-1.3	0.41
1416321_s_at	*PRELP*	Proline arginine-rich end leucine-rich repeat	-1.3	0.41
1452869_at	*PRPF38B*	PRP38 pre-mrna processing factor 38 (yeast) domain containing B	-1.3	0.41
1429169_at	*RBM3*	RNA binding motif protein 3	-1.3	0.41
1435933_at	*SCN2A1*	Sodium channel, voltage-gated, type II, alpha 1	-1.3	0.41
1432798_at	*SLC12A6*	Solute carrier family 12, member 6	-1.3	0.41
1429662_at	*SPATA1*	Spermatogenesis associated 1	-1.3	0.41
1441024_at	*STIM2*	Stromal interaction molecule 2	-1.3	0.41
1419088_at	*TIMP3*	Tissue inhibitor of metalloproteinase 3	-1.3	0.41

To be cont'd

Affymetrix ID	Gene symbol	Gene name	Signal log ratio	Fold change
1455162_at	*TTC39A*	Tetratricopeptide repeat domain 39A	-1.3	0.41
1434842_s_at	*UPF3B*	UPF3 regulator of nonsense transcripts homolog B (yeast)	-1.3	0.41
1460657_at	*WNT10A*	Wingless related MMTV integration site 10a	-1.3	0.41
1439073_at	*ZFP160*	Zinc finger protein 160	-1.3	0.41
1438343_at	*0610037L13RIK*	RIKEN cdna 0610037L13 gene	-1.2	0.44
1431818_at	*1700012B15RIK*	RIKEN cdna 1700012B15 gene	-1.2	0.44
1451415_at	*1810011O10RIK*	RIKEN cdna 1810011O10 gene	-1.2	0.44
1439495_at	*4933407H18RIK*	RIKEN cdna 4933407H18 gene	-1.2	0.44
1427371_at	*ABCA8A*	ATP-binding cassette, sub-family A (ABC1), member 8a	-1.2	0.44
1433598_at	*ARGLU1*	Arginine and glutamate rich 1	-1.2	0.44
1460736_at	*BOD1L*	Biorientation of chromosomes in cell division 1-like	-1.2	0.44
1455738_at	*CCDC55*	Coiled-coil domain containing 55	-1.2	0.44
1427138_at	*CCDC88C*	Coiled-coil domain containing 88C	-1.2	0.44
1435779_at	*CEP110*	Centrosomal protein 110	-1.2	0.44
1438109_at	*CLCA5*	Chloride channel calcium activated 5	-1.2	0.44
1460372_at	*DUOXA1*	Dual oxidase maturation factor 1	-1.2	0.44
1436329_at	*EGR3*	Early growth response 3	-1.2	0.44
1434605_at	*EIF5B*	Eukaryotic translation initiation factor 5B	-1.2	0.44
1439757_s_at	*EPHA4*	Eph receptor A4	-1.2	0.44
1428427_at	*FBXL2*	F-box and leucine-rich repeat protein 2	-1.2	0.44
1455337_at	*FGD4*	FYVE, rhogef and PH domain containing 4	-1.2	0.44
1453851_a_at	*GADD45G*	Growth arrest and DNA-damage-inducible 45 gamma	-1.2	0.44
1443939_at	*GM12824*	Predicted gene 12824	-1.2	0.44
1418301_at	*IRF6*	Interferon regulatory factor 6	-1.2	0.44
1425039_at	*ITGBL1*	Integrin, beta-like 1	-1.2	0.44
1427285_s_at	*MALAT1*	Metastasis associated lung adenocarcinoma transcript 1 (non-coding RNA)	-1.2	0.44
1436796_at	*MATR3*	Matrin 3	-1.2	0.44
1424123_at	*MFSD7C*	Major facilitator superfamily domain containing 7C	-1.2	0.44
1455238_at	*MUM1L1*	Melanoma associated antigen (mutated) 1-like 1	-1.2	0.44
1425153_at	*MYH2*	Myosin, heavy polypeptide 2, skeletal muscle, adult	-1.2	0.44
1435041_at	*MYL6*	Myosin, light polypeptide 6, alkali, smooth muscle and non-muscle	-1.2	0.44
1444980_at	*ONECUT2*	One cut domain, family member 2	-1.2	0.44
1453145_at	*PISD-PS3*	Phosphatidylserine decarboxylase, pseudogene 3	-1.2	0.44
1431110_at	*PLXDC2*	Plexin domain containing 2	-1.2	0.44
1426622_a_at	*QPCT*	Glutaminyl-peptide cyclotransferase (glutaminyl cyclase)	-1.2	0.44
1443715_at	*RBM24*	RNA binding motif protein 24	-1.2	0.44
1448201_at	*SFRP2*	Secreted frizzled-related protein 2	-1.2	0.44

To be cont'd

Affymetrix ID	Gene symbol	Gene name	Signal log ratio	Fold change
1415874_at	*SPRY1*	Sprouty homolog 1 (Drosophila)	-1.2	0.44
1420018_s_at	*TSPAN8*	Tetraspanin 8	-1.2	0.44
1455807_at	*TSPYL5*	Testis-specific protein, Y-encoded-like 5	-1.2	0.44
1460448_s_at	*TTC14*	Tetratricopeptide repeat domain 14	-1.2	0.44
1427263_at	*XIST*	Inactive X specific transcripts	-1.2	0.44
1454975_at	*ZFC3H1*	Zinc finger, C3H1-type containing	-1.2	0.44
1421139_a_at	*ZFP386*	Zinc finger protein 386 (Kruppel-like)	-1.2	0.44
1430886_at	*1700112E06RIK*	RIKEN cdna 1700112E06 gene	-1.1	0.47
1429953_at	*2210011C24RIK*	RIKEN cdna 2210011C24 gene	-1.1	0.47
1435794_at	*5031425E22RIK*	RIKEN cdna 5031425E22 gene	-1.1	0.47
1428220_at	*5730419I09RIK*	RIKEN cdna 5730419I09 gene	-1.1	0.47
1453658_at	*5830477G23RIK*	RIKEN cdna 5830477G23 gene	-1.1	0.47
1457987_at	*6030458C11RIK*	RIKEN cdna 6030458C11 gene	-1.1	0.47
1438720_at	*9330159F19RIK*	RIKEN cdna 9330159F19 gene	-1.1	0.47
1428387_at	*ACSL3*	Acyl-coa synthetase long-chain family member 3	-1.1	0.47
1448789_at	*ALDH1A3*	Aldehyde dehydrogenase family 1, subfamily A3	-1.1	0.47
1425677_a_at	*ANK1*	Ankyrin 1, erythroid	-1.1	0.47
1440193_at	*ANKRD12*	Ankyrin repeat domain 12	-1.1	0.47
1429247_at	*ANXA6*	Annexin A6	-1.1	0.47
1455396_at	*ATP8B1*	Atpase, class I, type 8B, member 1	-1.1	0.47
1450051_at	*ATRX*	Alpha thalassemia/mental retardation syndrome X-linked homolog (human)	-1.1	0.47
1437122_at	*BCL2*	B cell leukemia/lymphoma 2	-1.1	0.47
1448595_a_at	*BEX1*	Brain expressed gene 1	-1.1	0.47
1422912_at	*BMP4*	Bone morphogenetic protein 4	-1.1	0.47
1449345_at	*CCDC34*	Coiled-coil domain containing 34	-1.1	0.47
1453045_at	*CCDC41*	Coiled-coil domain containing 41	-1.1	0.47
1436572_at	*CCDC45*	Coiled-coil domain containing 45	-1.1	0.47
1443906_at	*CD55*	CD55 antigen	-1.1	0.47
1435005_at	*CENPE*	Centromere protein E	-1.1	0.47
1457823_at	*CYR61*	Cysteine rich protein 61	-1.1	0.47
1435357_at	*D4WSU53E*	DNA segment, Chr 4, Wayne State University 53, expressed	-1.1	0.47
1455361_at	*DGKB*	Diacylglycerol kinase, beta	-1.1	0.47
1436650_at	*FILIP1*	Filamin A interacting protein 1	-1.1	0.47
1437820_at	*FOXS1*	Forkhead box S1	-1.1	0.47
1451285_at	*FUS*	Fusion, derived from t(12;16) malignant liposarcoma (human)	-1.1	0.47
1440342_at	*G530011O06RIK*	RIKEN cdna G530011O06 gene	-1.1	0.47
1431687_at	*GMFB*	Glia maturation factor, beta	-1.1	0.47
1452239_at	*GT(ROSA)26SOR*	Gene trap ROSA 26, Philippe Soriano	-1.1	0.47
1418102_at	*HES1*	Hairy and enhancer of split 1 (Drosophila)	-1.1	0.47
1419302_at	*HEYL*	Hairy/enhancer-of-split related with YRPW motif-like	-1.1	0.47
1419519_at	*IGF1*	Insulin-like growth factor 1	-1.1	0.47

To be cont'd

Affymetrix ID	Gene symbol	Gene name	Signal log ratio	Fold change
1425454_a_at	*IL12A*	Interleukin 12a	-1.1	0.47
1454768_at	*KCNF1*	Potassium voltage-gated channel, subfamily F, member 1	-1.1	0.47
1447551_x_at	*LPHN3*	Latrophilin 3	-1.1	0.47
1429679_at	*LRRC17*	Leucine rich repeat containing 17	-1.1	0.47
1451498_at	*LRRC26*	Leucine rich repeat containing 26	-1.1	0.47
1453836_a_at	*MGLL*	Monoglyceride lipase	-1.1	0.47
1422597_at	*MMP15*	Matrix metallopeptidase 15	-1.1	0.47
1418388_s_at	*MPHOSPH8*	M-phase phosphoprotein 8	-1.1	0.47
1434830_at	*MXD1*	MAX dimerization protein 1	-1.1	0.47
1449586_at	*PKP1*	Plakophilin 1	-1.1	0.47
1430636_at	*PPP4R1L-PS*	Protein phosphatase 4, regulatory subunit 1-like, pseudogene	-1.1	0.47
1421354_at	*PRKG2*	Protein kinase, cgmp-dependent, type II	-1.1	0.47
1428172_at	*PRPF39*	PRP39 pre-mrna processing factor 39 homolog (yeast)	-1.1	0.47
1442148_at	*PSIP1*	PC4 and SFRS1 interacting protein 1	-1.1	0.47
1455358_at	*RBFOX1*	RNA binding protein, fox-1 homolog (C. Elegans) 1	-1.1	0.47
1451041_at	*ROCK2*	Rho-associated coiled-coil containing protein kinase 2	-1.1	0.47
1438076_at	*RPL30*	Ribosomal protein L30	-1.1	0.47
1420417_at	*SEMA3A*	Sema domain, immunoglobulin domain (Ig), short basic domain, secreted, (semaphorin) 3A	-1.1	0.47
1451031_at	*SFRP4*	Secreted frizzled-related protein 4	-1.1	0.47
1456914_at	*SLC16A4*	Solute carrier family 16 (monocarboxylic acid transporters), member 4	-1.1	0.47
1443749_x_at	*SLC1A3*	Solute carrier family 1 (glial high affinity glutamate transporter), member 3	-1.1	0.47
1424452_at	*SLTM*	SAFB-like, transcription modulator	-1.1	0.47
1437658_a_at	*SNHG1*	Small nucleolar RNA host gene (non-protein coding) 1	-1.1	0.47
1427500_at	*SPCS1*	Signal peptidase complex subunit 1 homolog (S. Cerevisiae)	-1.1	0.47
1430271_x_at	*TAF1D*	TATA box binding protein (Tbp)-associated factor, RNA polymerase I, D	-1.1	0.47
1423505_at	*TAGLN*	Transgelin	-1.1	0.47
1428467_at	*TARDBP*	TAR DNA binding protein	-1.1	0.47
1435585_at	*TCEAL7*	Transcription elongation factor A (SII)-like 7	-1.1	0.47
1437634_at	*THOC2*	THO complex 2	-1.1	0.47
1418398_a_at	*TSPAN32*	Tetraspanin 32	-1.1	0.47
1453058_at	*WDR5B*	WD repeat domain 5B	-1.1	0.47
1436396_at	*WDR60*	WD repeat domain 60	-1.1	0.47
1431297_a_at	*4933436C20RIK*	RIKEN cdna 4933436C20 gene	-1.0	0.50
1429987_at	*9930013L23RIK*	RIKEN cdna 9930013L23 gene	-1.0	0.50
1429214_at	*ADAMTSL2*	ADAMTS-like 2	-1.0	0.50
1435160_at	*AHSA2*	AHA1, activator of heat shock protein atpase 2	-1.0	0.50
1426301_at	*ALCAM*	Activated leukocyte cell adhesion molecule	-1.0	0.50

To be cont'd

Affymetrix ID	Gene symbol	Gene name	Signal log ratio	Fold change
1452104_at	*ARL16*	ADP-ribosylation factor-like 16	-1.0	0.50
1433863_at	*BTF3*	Basic transcription factor 3	-1.0	0.50
1456224_x_at	*CAGE1*	Cancer antigen 1	-1.0	0.50
1417420_at	*CCND1*	Cyclin D1	-1.0	0.50
1423622_a_at	*CCNL1*	Cyclin L1	-1.0	0.50
1433450_at	*CDK5R1*	Cyclin-dependent kinase 5, regulatory subunit 1 (p35)	-1.0	0.50
1452991_at	*CHD2*	Chromodomain helicase DNA binding protein 2	-1.0	0.50
1445194_at	*CNKSR2*	Connector enhancer of kinase suppressor of Ras 2	-1.0	0.50
1446326_at	*COL1A2*	Collagen, type I, alpha 2	-1.0	0.50
1434667_at	*COL8A2*	Collagen, type VIII, alpha 2	-1.0	0.50
1437051_at	*DFFB*	DNA fragmentation factor, beta subunit	-1.0	0.50
1451912_a_at	*FGFRL1*	Fibroblast growth factor receptor-like 1	-1.0	0.50
1452799_at	*FGGY*	FGGY carbohydrate kinase domain containing	-1.0	0.50
1445534_at	*FLNB*	Filamin, beta	-1.0	0.50
1433640_at	*FUBP1*	Far upstream element (FUSE) binding protein 1	-1.0	0.50
1448700_at	*G0S2*	G0/G1 switch gene 2	-1.0	0.50
1418007_at	*GCFC1*	GC-rich sequence DNA-binding factor 1	-1.0	0.50
1435695_a_at	*GGCT*	Gamma-glutamyl cyclotransferase	-1.0	0.50
1428301_at	*GM10128*	Predicted gene 10128	-1.0	0.50
1428250_at	*GPR30*	G protein-coupled receptor 30	-1.0	0.50
1418349_at	*HBEGF*	Heparin-binding EGF-like growth factor	-1.0	0.50
1431777_a_at	*HMGN3*	High mobility group nucleosomal binding domain 3	-1.0	0.50
1455740_at	*HNRNPA1*	Heterogeneous nuclear ribonucleoprotein A1	-1.0	0.50
1427433_s_at	*HOXA3*	Homeobox A3	-1.0	0.50
1435332_at	*HTR7*	5-hydroxytryptamine (serotonin) receptor 7	-1.0	0.50
1423104_at	*IRS1*	Insulin receptor substrate 1	-1.0	0.50
1450029_s_at	*ITGA9*	Integrin alpha 9	-1.0	0.50
1428615_at	*LPAR6*	Lysophosphatidic acid receptor 6	-1.0	0.50
1417511_at	*LYAR*	Ly1 antibody reactive clone	-1.0	0.50
1435514_at	*LZTFL1*	Leucine zipper transcription factor-like 1	-1.0	0.50
1434364_at	*MAP3K14*	Mitogen-activated protein kinase kinase kinase 14	-1.0	0.50
1456927_at	*MAST2*	Microtubule associated serine/threonine kinase 2	-1.0	0.50
1449965_at	*MCPT8*	Mast cell protease 8	-1.0	0.50
1421028_a_at	*MEF2C*	Myocyte enhancer factor 2C	-1.0	0.50
1452349_x_at	*MNDA*	Myeloid cell nuclear differentiation antigen	-1.0	0.50
1434194_at	*MTAP2*	Microtubule-associated protein 2	-1.0	0.50
1452670_at	*MYL9*	Myosin, light polypeptide 9, regulatory	-1.0	0.50
1441111_at	*MYLK4*	Myosin light chain kinase family, member 4	-1.0	0.50

To be cont'd

Affymetrix ID	Gene symbol	Gene name	Signal log ratio	Fold change
1424543_at	*NCK1*	Non-catalytic region of tyrosine kinase adaptor protein 1	-1.0	0.50
1435649_at	*NEXN*	Nexilin	-1.0	0.50
1434275_at	*NKD2*	Naked cuticle 2 homolog (Drosophila)	-1.0	0.50
1423249_at	*NKTR*	Natural killer tumor recognition sequence	-1.0	0.50
1418355_at	*NUCB2*	Nucleobindin 2	-1.0	0.50
1419665_a_at	*NUPR1*	Nuclear protein 1	-1.0	0.50
1423915_at	*OLFML2B*	Olfactomedin-like 2B	-1.0	0.50
1420797_at	*OTOG*	Otogelin	-1.0	0.50
1419767_at	*PADI3*	Peptidyl arginine deiminase, type III	-1.0	0.50
1440635_at	*PALLD*	Palladin, cytoskeletal associated protein	-1.0	0.50
1456532_at	*PDGFD*	Platelet-derived growth factor, D polypeptide	-1.0	0.50
1421413_a_at	*PDLIM5*	PDZ and LIM domain 5	-1.0	0.50
1435353_a_at	*PISD-PS1*	Phosphatidylserine decarboxylase, pseudogene 1	-1.0	0.50
1443282_at	*PRPF38A*	PRP38 pre-mrna processing factor 38 (yeast) domain containing A	-1.0	0.50
1435316_at	*PSMA6*	Proteasome (prosome, macropain) subunit, alpha type 6	-1.0	0.50
1428789_at	*RALGPS2*	Ral GEF with PH domain and SH3 binding motif 2	-1.0	0.50
1424470_a_at	*RAPGEF3*	Rap guanine nucleotide exchange factor (GEF) 3	-1.0	0.50
1443033_at	*RBM14*	RNA binding motif protein 14	-1.0	0.50
1435391_at	*RBM33*	RNA binding motif protein 33	-1.0	0.50
1432232_at	*RCOR3*	REST corepressor 3	-1.0	0.50
1421565_at	*ROBO3*	Roundabout homolog 3 (Drosophila)	-1.0	0.50
1426123_a_at	*RRBP1*	Ribosome binding protein 1	-1.0	0.50
1451640_a_at	*RSRC2*	Arginine/serine-rich coiled-coil 2	-1.0	0.50
1427988_s_at	*SAFB2*	Scaffold attachment factor B2	-1.0	0.50
1460603_at	*SAMD9L*	Sterile alpha motif domain containing 9-like	-1.0	0.50
1458813_at	*SCN5A*	Sodium channel, voltage-gated, type V, alpha	-1.0	0.50
1416778_at	*SDPR*	Serum deprivation response	-1.0	0.50
1448415_a_at	*SEMA3B*	Sema domain, immunoglobulin domain (Ig), short basic domain, secreted, (semaphorin) 3B	-1.0	0.50
1459903_at	*SEMA7A*	Sema domain, immunoglobulin domain (Ig), and GPI membrane anchor, (semaphorin) 7A	-1.0	0.50
1457266_at	*SLC38A6*	Solute carrier family 38, member 6	-1.0	0.50
1427275_at	*SMC4*	Structural maintenance of chromosomes 4	-1.0	0.50
1448829_at	*SMC6*	Structural maintenance of chromosomes 6	-1.0	0.50
1427134_at	*SREK1*	Splicing regulatory glutamine/lysine-rich protein 1	-1.0	0.50
1427430_at	*SUCO*	SUN domain containing ossification factor	-1.0	0.50
1438303_at	*TGFB2*	Transforming growth factor, beta 2	-1.0	0.50

To be cont'd

Affymetrix ID	Gene symbol	Gene name	Signal log ratio	Fold change
1449969_at	*TMOD4*	Tropomodulin 4	-1.0	0.50
1452027_a_at	*TRP63*	Transformation related protein 63	-1.0	0.50
1431279_s_at	*TTLL5*	Tubulin tyrosine ligase-like family, member 5	-1.0	0.50
1449682_s_at	*TUBB2B*	Tubulin, beta 2B class IIB	-1.0	0.50
1460272_at	*UBL4B*	Ubiquitin-like 4B	-1.0	0.50
1430568_at	*ZC3H13*	Zinc finger CCCH type containing 13	-1.0	0.50
1455092_at	*ZFP207*	Zinc finger protein 207	-1.0	0.50
1437128_a_at	*ZFP945*	Zinc finger protein 945	-1.0	0.50
1432001_at	*ZMYND17*	Zinc finger, MYND domain containing 17	-1.0	0.50
1424999_at	*ZNRD1AS*	ZNRD1 antisense RNA	-1.0	0.50

Table 28. List of up-regulated genes in C2C12 myotubes upon treatment with 300 mM ethanol

Affymetrix ID	Gene symbol	Gene name	Signal log ratio	Fold change
1433607_at	*CBLN4*	Cerebellin 4 precursor protein	3.4	10.56
1434526_at	*EPHX4*	Epoxide hydrolase 4	1.8	3.48
1457775_at	*SACS*	Sacsin	1.8	3.48
1444176_at	*ATP6V0D2*	Atpase, H+ transporting, lysosomal V0 subunit D2	1.5	2.83
1417231_at	*CLDN2*	Claudin 2	1.4	2.64
1419209_at	*CXCL1*	Chemokine (C-X-C motif) ligand 1	1.2	2.30
1419728_at	*CXCL5*	Chemokine (C-X-C motif) ligand 5	1.2	2.30
1419145_at	*SMTNL1*	Smoothelin-like 1	1.2	2.30
1436109_at	*AI317395*	Expressed sequence AI317395	1.1	2.14
1451280_at	*ARPP21*	Cyclic AMP-regulated phosphoprotein, 21	1.1	2.14
1421228_at	*CCL7*	Chemokine (C-C motif) ligand 7	1.1	2.14
1417408_at	*F3*	Coagulation factor III	1.1	2.14
1416676_at	*KNG1*	Kininogen 1	1.1	2.14
1453898_at	*NMRK2*	Nicotinamide riboside kinase 2	1.1	2.14
1424443_at	*TM6SF1*	Transmembrane 6 superfamily member 1	1.0	2.00
1428722_at	*CKMT2*	Creatine kinase, mitochondrial 2	0.9	1.87
1453822_at	*DNALC1*	Dynein, axonemal, light chain 1	0.9	1.87
1415904_at	*LPL*	Lipoprotein lipase	0.9	1.87
1452031_at	*SLC1A3*	Solute carrier family 1 (glial high affinity glutamate transporter), member 3	0.9	1.87
1417426_at	*SRGN*	Serglycin	0.9	1.87
1416713_at	*TPPP3*	Tubulin polymerization-promoting protein family member 3	0.9	1.87
1422153_a_at	*ASB11*	Ankyrin repeat and SOCS box-containing 11	0.8	1.74
1418174_at	*DBP*	D site albumin promoter binding protein	0.8	1.74
1438953_at	*FIGF*	C-fos induced growth factor	0.8	1.74
1442884_at	*HGF*	Hepatocyte growth factor	0.8	1.74
1443921_at	*RANBP3L*	RAN binding protein 3-like	0.8	1.74
1451204_at	*SCARA5*	Scavenger receptor class A, member 5 (putative)	0.8	1.74
1429961_at	*TSACC*	TSSK6 activating co-chaperone	0.8	1.74
1451780_at	*BLNK*	B cell linker	0.7	1.62
1448649_at	*ENPEP*	Glutamyl aminopeptidase	0.7	1.62
1416023_at	*FABP3*	Fatty acid binding protein 3, muscle and heart	0.7	1.62
1422540_at	*FBLN1*	Fibulin 1	0.7	1.62
1450440_at	*GFRA1*	Glial cell line derived neurotrophic factor family receptor alpha 1	0.7	1.62
1448269_a_at	*KLHL13*	Kelch-like 13 (Drosophila)	0.7	1.62

To be cont'd

Affymetrix ID	Gene symbol	Gene name	Signal log ratio	Fold change
1428083_at	*NEAT1*	Nuclear paraspeckle assembly transcript 1 (non-protein coding)	0.7	1.62
1418187_at	*RAMP2*	Receptor (calcitonin) activity modifying protein 2	0.7	1.62
1448529_at	*THBD*	Thrombomodulin	0.7	1.62
1450004_at	*TSLP*	Thymic stromal lymphopoietin	0.7	1.62
1453024_at	*WDR37*	WD repeat domain 37	0.7	1.62
1427371_at	*ABCA8A*	ATP-binding cassette, sub-family A (ABC1), member 8a	0.6	1.52
1417946_at	*ABHD3*	Abhydrolase domain containing 3	0.6	1.52
1416203_at	*AQP1*	Aquaporin 1	0.6	1.52
1419684_at	*CCL8*	Chemokine (C-C motif) ligand 8	0.6	1.52
1449402_at	*CHST7*	Carbohydrate (N-acetylglucosamino) sulfotransferase 7	0.6	1.52
1419477_at	*CLEC2D*	C-type lectin domain family 2, member d	0.6	1.52
1415857_at	*EMB*	Embigin	0.6	1.52
1436536_at	*EXOC3L*	Exocyst complex component 3-like	0.6	1.52
1430986_at	*FARSB*	Phenylalanyl-trna synthetase, beta subunit	0.6	1.52
1417872_at	*FHL1*	Four and a half LIM domains 1	0.6	1.52
1435749_at	*GDA*	Guanine deaminase	0.6	1.52
1439837_at	*GIGYF2*	GRB10 interacting GYF protein 2	0.6	1.52
1423091_a_at	*GPM6B*	Glycoprotein m6b	0.6	1.52
1428361_x_at	*HBA-A1*	Hemoglobin alpha, adult chain 1	0.6	1.52
1449038_at	*HSD11B1*	Hydroxysteroid 11-beta dehydrogenase 1	0.6	1.52
1423608_at	*ITM2A*	Integral membrane protein 2A	0.6	1.52
1451762_a_at	*KIF1B*	Kinesin family member 1B	0.6	1.52
1434877_at	*NPTX1*	Neuronal pentraxin 1	0.6	1.52
1417653_at	*PVALB*	Parvalbumin	0.6	1.52
1419247_at	*RGS2*	Regulator of G-protein signaling 2	0.6	1.52
1416523_at	*RNASE1*	Ribonuclease, rnase A family, 1 (pancreatic)	0.6	1.52
1441370_at	*TMCC1*	Transmembrane and coiled coil domains 1	0.6	1.52
1425098_at	*ZFP106*	Zinc finger protein 106	0.6	1.52
1453339_at	*1700008I05RIK*	RIKEN cdna 1700008I05 gene	0.5	1.41
1442376_at	*ABLIM1*	Actin-binding LIM protein 1	0.5	1.41
1423556_at	*AKR1B7*	Aldo-keto reductase family 1, member B7	0.5	1.41
1418979_at	*AKR1C14*	Aldo-keto reductase family 1, member C14	0.5	1.41
1419621_at	*ANKRD2*	Ankyrin repeat domain 2 (stretch responsive muscle)	0.5	1.41
1460036_at	*AP1S2*	Adaptor-related protein complex 1, sigma 2 subunit	0.5	1.41
1454822_x_at	*APCDD1*	Adenomatosis polyposis coli down-regulated 1	0.5	1.41

To be cont'd

Affymetrix ID	Gene symbol	Gene name	Signal log ratio	Fold change
1417789_at	*CCL11*	Chemokine (C-C motif) ligand 11	0.5	1.41
1419693_at	*COLEC12*	Collectin sub-family member 12	0.5	1.41
1455660_at	*CSF2RB*	Colony stimulating factor 2 receptor, beta, low-affinity (granulocyte-macrophage)	0.5	1.41
1443814_x_at	*CTSH*	Cathepsin H	0.5	1.41
1448792_a_at	*CYP2F2*	Cytochrome P450, family 2, subfamily f, polypeptide 2	0.5	1.41
1451389_at	*DNAJC24*	Dnaj (Hsp40) homolog, subfamily C, member 24	0.5	1.41
1448136_at	*ENPP2*	Ectonucleotide pyrophosphatase/phosphodiesterase 2	0.5	1.41
1454965_at	*FAM171B*	Family with sequence similarity 171, member B	0.5	1.41
1448494_at	*GAS1*	Growth arrest specific 1	0.5	1.41
1430834_at	*GPRIN3*	GPRIN family member 3	0.5	1.41
1425357_a_at	*GREM1*	Gremlin 1	0.5	1.41
1433944_at	*HECTD2*	HECT domain containing 2	0.5	1.41
1452014_a_at	*IGF1*	Insulin-like growth factor 1	0.5	1.41
1449983_a_at	*NQO2*	NAD(P)H dehydrogenase, quinone 2	0.5	1.41
1456925_at	*P2RX6*	Purinergic receptor P2X, ligand-gated ion channel, 6	0.5	1.41
1422474_at	*PDE4B*	Phosphodiesterase 4B, camp specific	0.5	1.41
1421979_at	*PHEX*	Phosphate regulating gene with homologies to endopeptidases on the X chromosome (hypophosphatemia, vitamin D resistant rickets)	0.5	1.41
1416166_a_at	*PRDX4*	Peroxiredoxin 4	0.5	1.41
1420349_at	*PTGFR*	Prostaglandin F receptor	0.5	1.41
1448254_at	*PTN*	Pleiotrophin	0.5	1.41
1440769_at	*SELRC1*	Sel1 repeat containing 1	0.5	1.41
1457275_at	*SYNM*	Synemin, intermediate filament protein	0.5	1.41
1460353_at	*TMEM48*	Transmembrane protein 48	0.5	1.41
1422536_at	*TNNI3*	Troponin I, cardiac 3	0.5	1.41
1447713_at	*TPM1*	Tropomyosin 1, alpha	0.5	1.41
1434340_at	*UQCR10*	Ubiquinol-cytochrome c reductase, complex III subunit X	0.5	1.41
1451314_a_at	*VCAM1*	Vascular cell adhesion molecule 1	0.5	1.41

Table 29. List of down-regulated genes in C2C12 myotubes upon treatment with 300 mM ethanol

Affymetrix ID	Gene symbol	Gene name	Signal log ratio	Fold change
1417156_at	*KRT19*	Keratin 19	-2.2	0.22
1452732_at	*ASPRV1*	Aspartic peptidase, retroviral-like 1	-1.7	0.31
1436218_at	*LGR6*	Leucine-rich repeat-containing G protein-coupled receptor 6	-1.6	0.33
1455948_x_at	*MATN3*	Matrilin 3	-1.5	0.35
1453588_at	*CAR3*	Carbonic anhydrase 3	-1.4	0.38
1427013_at	*CAR9*	Carbonic anhydrase 9	-1.4	0.38
1425589_at	*HSD17B13*	Hydroxysteroid (17-beta) dehydrogenase 13	-1.2	0.44
1437025_at	*CD28*	CD28 antigen	-1.1	0.47
1438540_at	*COL25A1*	Collagen, type XXV, alpha 1	-1.1	0.47
1429637_at	*FAM198B*	Family with sequence similarity 198, member B	-1.1	0.47
1422462_at	*UBE2T*	Ubiquitin-conjugating enzyme E2T (putative)	-1.1	0.47
1418441_at	*COL8A1*	Collagen, type VIII, alpha 1	-1.0	0.50
1416641_at	*LIG1*	Ligase I, DNA, ATP-dependent	-1.0	0.50
1418422_at	*SERPINB9G*	Serine (or cysteine) peptidase inhibitor, clade B, member 9g	-1.0	0.50
1449033_at	*TNFRSF11B*	Tumor necrosis factor receptor superfamily, member 11b (osteoprotegerin)	-1.0	0.50
1427537_at	*EPPK1*	Epiplakin 1	-0.9	0.54
1421041_s_at	*GSTA1*	Glutathione S-transferase, alpha 1 (Ya)	-0.9	0.54
1456772_at	*NCF1*	Neutrophil cytosolic factor 1	-0.9	0.54
1429699_at	*OXSM*	3-oxoacyl-ACP synthase, mitochondrial	-0.9	0.54
1422924_at	*TNFSF9*	Tumor necrosis factor (ligand) superfamily, member 9	-0.9	0.54
1416077_at	*ADM*	Adrenomedullin	-0.8	0.57
1424518_at	*APOL9B*	Apolipoprotein L 9b	-0.8	0.57
1418304_at	*CDHR1*	Cadherin-related family member 1	-0.8	0.57
1456084_x_at	*FMOD*	Fibromodulin	-0.8	0.57
1419139_at	*GDF5*	Growth differentiation factor 5	-0.8	0.57
1443939_at	*GM12824*	Predicted gene 12824	-0.8	0.57
1450029_s_at	*ITGA9*	Integrin alpha 9	-0.8	0.57
1417812_a_at	*LAMB3*	Laminin, beta 3	-0.8	0.57
1448978_at	*NGEF*	Neuronal guanine nucleotide exchange factor	-0.8	0.57
1421429_a_at	*NPNT*	Nephronectin	-0.8	0.57
1453069_at	*PIK3CB*	Phosphatidylinositol 3-kinase, catalytic, beta polypeptide	-0.8	0.57
1421430_at	*RAD51L1*	RAD51-like 1 (S. Cerevisiae)	-0.8	0.57
1459415_at	*SPECC1*	Sperm antigen with calponin homology and coiled-coil domains 1	-0.8	0.57

To be cont'd

Affymetrix ID	Gene symbol	Gene name	Signal log ratio	Fold change
1446072_at	*4632415L05RIK*	RIKEN cdna 4632415L05 gene	-0.7	0.62
1437841_x_at	*CSDC2*	Cold shock domain containing C2, RNA binding	-0.7	0.62
1422534_at	*CYP51*	Cytochrome P450, family 51	-0.7	0.62
1440866_at	*EIF2AK2*	Eukaryotic translation initiation factor 2-alpha kinase 2	-0.7	0.62
1452799_at	*FGGY*	FGGY carbohydrate kinase domain containing	-0.7	0.62
1456480_at	*FRY*	Furry homolog (Drosophila)	-0.7	0.62
1440021_at	*GPR20*	G protein-coupled receptor 20	-0.7	0.62
1417275_at	*MAL*	Myelin and lymphocyte protein, T cell differentiation protein	-0.7	0.62
1434830_at	*MXD1*	MAX dimerization protein 1	-0.7	0.62
1434628_a_at	*RHPN2*	Rhophilin, Rho gtpase binding protein 2	-0.7	0.62
1418423_s_at	*SERPINB9E*	Serine (or cysteine) peptidase inhibitor, clade B, member 9e	-0.7	0.62
1451601_a_at	*SPNS2*	Spinster homolog 2	-0.7	0.62
1423280_at	*STMN2*	Stathmin-like 2	-0.7	0.62
1442914_at	*TMF1*	TATA element modulatory factor 1	-0.7	0.62
1456344_at	*TNC*	Tenascin C	-0.7	0.62
1460657_at	*WNT10A*	Wingless related MMTV integration site 10a	-0.7	0.62
1457682_at	*ARHGAP42*	Rho gtpase activating protein 42	-0.6	0.66
1448261_at	*CDH1*	Cadherin 1	-0.6	0.66
1423669_at	*COL1A1*	Collagen, type I, alpha 1	-0.6	0.66
1429210_at	*COL23A1*	Collagen, type XXIII, alpha 1	-0.6	0.66
1416382_at	*CTSC*	Cathepsin C	-0.6	0.66
1428347_at	*CYFIP2*	Cytoplasmic FMR1 interacting protein 2	-0.6	0.66
1418129_at	*DHCR24*	24-dehydrocholesterol reductase	-0.6	0.66
1455108_at	*EIF4E2*	Eukaryotic translation initiation factor 4E member 2	-0.6	0.66
1417978_at	*EIF4E3*	Eukaryotic translation initiation factor 4E member 3	-0.6	0.66
1433796_at	*ENDOD1*	Endonuclease domain containing 1	-0.6	0.66
1435436_at	*EPAS1*	Endothelial PAS domain protein 1	-0.6	0.66
1455872_at	*FAM167A*	Family with sequence similarity 167, member A	-0.6	0.66
1449976_a_at	*GPR35*	G protein-coupled receptor 35	-0.6	0.66
1455014_at	*HINT3*	Histidine triad nucleotide binding protein 3	-0.6	0.66
1457248_x_at	*HSD17B7*	Hydroxysteroid (17-beta) dehydrogenase 7	-0.6	0.66
1424949_at	*HUWE1*	HECT, UBA and WWE domain containing 1	-0.6	0.66
1439954_at	*LEMD1*	LEM domain containing 1	-0.6	0.66
1424718_at	*MAPT*	Microtubule-associated protein tau	-0.6	0.66
1428835_at	*MYH14*	Myosin, heavy polypeptide 14	-0.6	0.66

To be cont'd

Affymetrix ID	Gene symbol	Gene name	Signal log ratio	Fold change
1425153_at	*MYH2*	Myosin, heavy polypeptide 2, skeletal muscle, adult	-0.6	0.66
1418932_at	*NFIL3*	Nuclear factor, interleukin 3, regulated	-0.6	0.66
1418252_at	*PADI2*	Peptidyl arginine deiminase, type II	-0.6	0.66
1441026_at	*PARP4*	Poly (ADP-ribose) polymerase family, member 4	-0.6	0.66
1427893_a_at	*PMVK*	Phosphomevalonate kinase	-0.6	0.66
1455450_at	*PTPN3*	Protein tyrosine phosphatase, non-receptor type 3	-0.6	0.66
1456980_at	*RAB44*	RAB44, member RAS oncogene family	-0.6	0.66
1430534_at	*RNASE6*	Ribonuclease, rnase A family, 6	-0.6	0.66
1442786_s_at	*RUFY3*	RUN and FYVE domain containing 3	-0.6	0.66
1419238_at	*ABCA7*	ATP-binding cassette, sub-family A (ABC1), member 7	-0.5	0.71
1434013_at	*ABLIM3*	Actin binding LIM protein family, member 3	-0.5	0.71
1449383_at	*ADSSL1*	Adenylosuccinate synthetase like 1	-0.5	0.71
1437373_at	*AEN*	Apoptosis enhancing nuclease	-0.5	0.71
1441962_at	*ALOX5*	Arachidonate 5-lipoxygenase	-0.5	0.71
1436181_at	*ASAP2*	Arfgap with SH3 domain, ankyrin repeat and PH domain 2	-0.5	0.71
1451539_at	*BAIAP2L1*	BAI1-associated protein 2-like 1	-0.5	0.71
1417419_at	*CCND1*	Cyclin D1	-0.5	0.71
1421295_at	*CHRDL1*	Chordin-like 1	-0.5	0.71
1429574_at	*CLIC3*	Chloride intracellular channel 3	-0.5	0.71
1446326_at	*COL1A2*	Collagen, type I, alpha 2	-0.5	0.71
1448619_at	*DHCR7*	7-dehydrocholesterol reductase	-0.5	0.71
1448390_a_at	*DHRS3*	Dehydrogenase/reductase (SDR family) member 3	-0.5	0.71
1433525_at	*EDNRA*	Endothelin receptor type A	-0.5	0.71
1418648_at	*EGLN3*	EGL nine homolog 3 (C. Elegans)	-0.5	0.71
1437071_at	*EIF1AX*	Eukaryotic translation initiation factor 1A, X-linked	-0.5	0.71
1427225_at	*EPN2*	Epsin 2	-0.5	0.71
1424919_at	*ERBB2*	V-erb-b2 erythroblastic leukemia viral oncogene homolog 2, neuro/glioblastoma derived oncogene homolog (avian)	-0.5	0.71
1447685_x_at	*ETS2*	E26 avian leukemia oncogene 2, 3' domain	-0.5	0.71
1416021_a_at	*FABP5*	Fatty acid binding protein 5, epidermal	-0.5	0.71
1436403_at	*FAM171A2*	Family with sequence similarity 171, member A2	-0.5	0.71
1418349_at	*HBEGF*	Heparin-binding EGF-like growth factor	-0.5	0.71
1416481_s_at	*HIGD1A*	HIG1 domain family, member 1A	-0.5	0.71

To be cont'd

Affymetrix ID	Gene symbol	Gene name	Signal log ratio	Fold change
1425553_s_at	*HIP1R*	Huntingtin interacting protein 1 related	-0.5	0.71
1422155_at	*HIST2H3C2*	Histone cluster 2, H3c2	-0.5	0.71
1433685_a_at	*HJURP*	Holliday junction recognition protein	-0.5	0.71
1425607_at	*HLCS*	Holocarboxylase synthetase (biotin-[propriony-Coenzyme A-carboxylase (ATP-hydrolysing)] ligase)	-0.5	0.71
1422870_at	*HOXC4*	Homeobox C4	-0.5	0.71
1458268_s_at	*IGFBP3*	Insulin-like growth factor binding protein 3	-0.5	0.71
1418983_at	*INADL*	Inad-like (Drosophila)	-0.5	0.71
1449082_at	*MFAP5*	Microfibrillar associated protein 5	-0.5	0.71
1450391_a_at	*MGLL*	Monoglyceride lipase	-0.5	0.71
1448553_at	*MYH7*	Myosin, heavy polypeptide 7, cardiac muscle, beta	-0.5	0.71
1441111_at	*MYLK4*	Myosin light chain kinase family, member 4	-0.5	0.71
1449550_at	*MYO1C*	Myosin IC	-0.5	0.71
1421525_a_at	*NAIP5*	NLR family, apoptosis inhibitory protein 5	-0.5	0.71
1450337_a_at	*NEK8*	NIMA (never in mitosis gene a)-related expressed kinase 8	-0.5	0.71
1423967_at	*PALM*	Paralemmin	-0.5	0.71
1423282_at	*PITPNA*	Phosphatidylinositol transfer protein, alpha	-0.5	0.71
1457252_x_at	*PLD2*	Phospholipase D2	-0.5	0.71
1447807_s_at	*PLEKHH1*	Pleckstrin homology domain containing, family H (with myth4 domain) member 1	-0.5	0.71
1416322_at	*PRELP*	Proline arginine-rich end leucine-rich repeat	-0.5	0.71
1418540_a_at	*PTPRE*	Protein tyrosine phosphatase, receptor type, E	-0.5	0.71
1420842_at	*PTPRF*	Protein tyrosine phosphatase, receptor type, F	-0.5	0.71
1424556_at	*PYCR1*	Pyrroline-5-carboxylate reductase 1	-0.5	0.71
1441964_at	*QK*	Quaking	-0.5	0.71
1436103_at	*RAB3IP*	RAB3A interacting protein	-0.5	0.71
1455750_at	*RALGAPA2*	Ral gtpase activating protein, alpha subunit 2 (catalytic)	-0.5	0.71
1434684_at	*RIN3*	Ras and Rab interactor 3	-0.5	0.71
1459897_a_at	*SBSN*	Suprabasin	-0.5	0.71
1415824_at	*SCD2*	Stearoyl-Coenzyme A desaturase 2	-0.5	0.71
1427417_at	*SCML4*	Sex comb on midleg-like 4 (Drosophila)	-0.5	0.71
1448415_a_at	*SEMA3B*	Sema domain, immunoglobulin domain (Ig), short basic domain, secreted, (semaphorin) 3B	-0.5	0.71
1426318_at	*SERPINB1B*	Serine (or cysteine) peptidase inhibitor, clade B, member 1b	-0.5	0.71

To be cont'd

Affymetrix ID	Gene symbol	Gene name	Signal log ratio	Fold change
1453004_at	*SLC22A23*	Solute carrier family 22, member 23	-0.5	0.71
1436279_at	*SLC26A7*	Solute carrier family 26, member 7	-0.5	0.71
1438115_a_at	*SLC9A3R1*	Solute carrier family 9 (sodium/hydrogen exchanger), member 3 regulator 1	-0.5	0.71
1422103_a_at	*STAT5B*	Signal transducer and activator of transcription 5B	-0.5	0.71
1419447_s_at	*TBC1D1*	TBC1 domain family, member 1	-0.5	0.71
1433471_at	*TCF7*	Transcription factor 7, T cell specific	-0.5	0.71
1429355_at	*TEKT5*	Tektin 5	-0.5	0.71
1418057_at	*TIAM1*	T cell lymphoma invasion and metastasis 1	-0.5	0.71
1449334_at	*TIMP3*	Tissue inhibitor of metalloproteinase 3	-0.5	0.71
1416571_at	*TRAF4*	TNF receptor associated factor 4	-0.5	0.71
1435170_at	*TSR2*	TSR2, 20S rrna accumulation, homolog (S. Cerevisiae)	-0.5	0.71
1428992_at	*UNC13D*	Unc-13 homolog D (C. Elegans)	-0.5	0.71

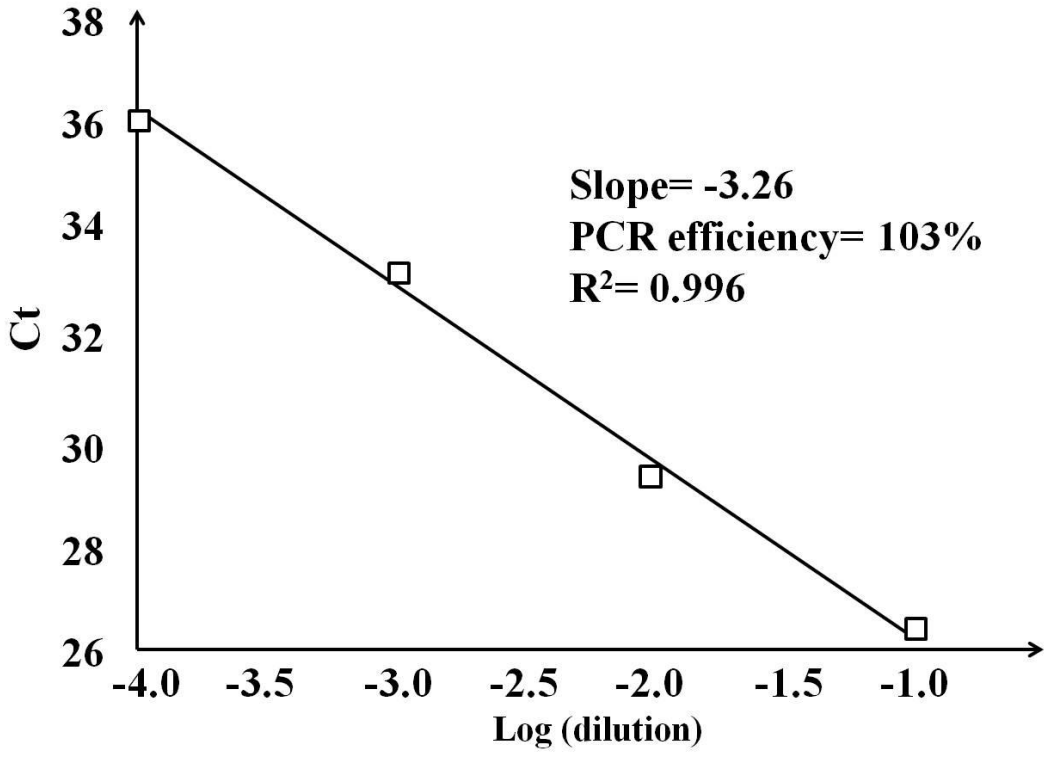

Figure 45. Typical qRT-PCR standard curve

Typical standard curve shown here was generated from four 10-fold dilutions of cDNA samples prepared from Caco-2 cell lysates, using specific primers for *GAPDH*. Each data point represents the value of one individual sample.

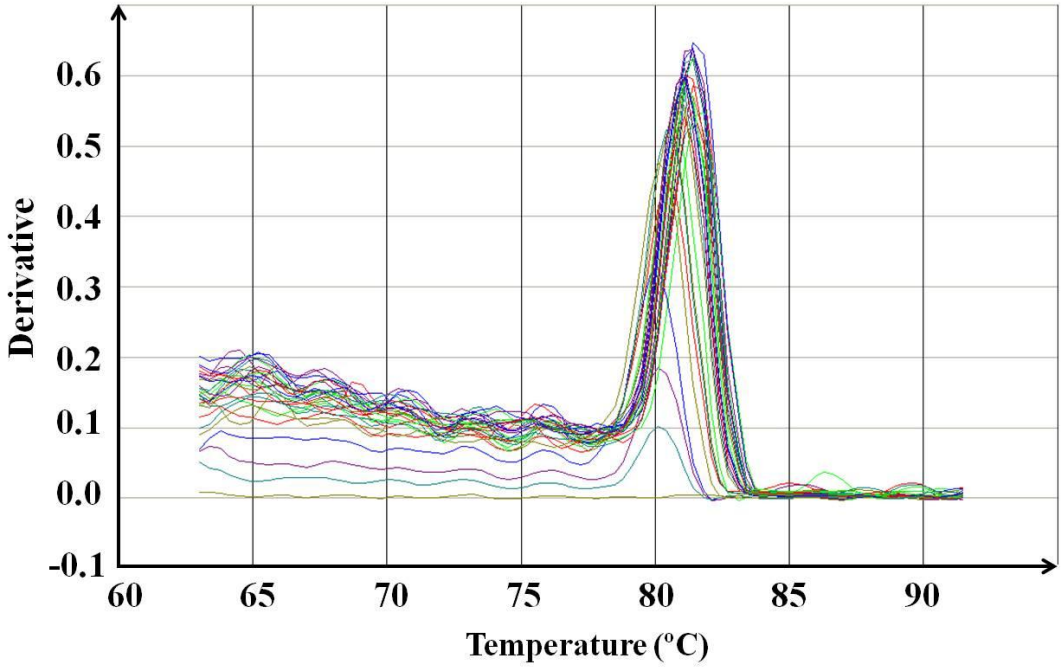

Figure 46. Typical qRT-PCR dissociation curves

The dissociation curves of 30 samples shown here were generated from qRT-PCR at the end of 40 cycles using specific primers for *GAPDH*. cDNA samples were prepared from Caco-2 cell lysates.

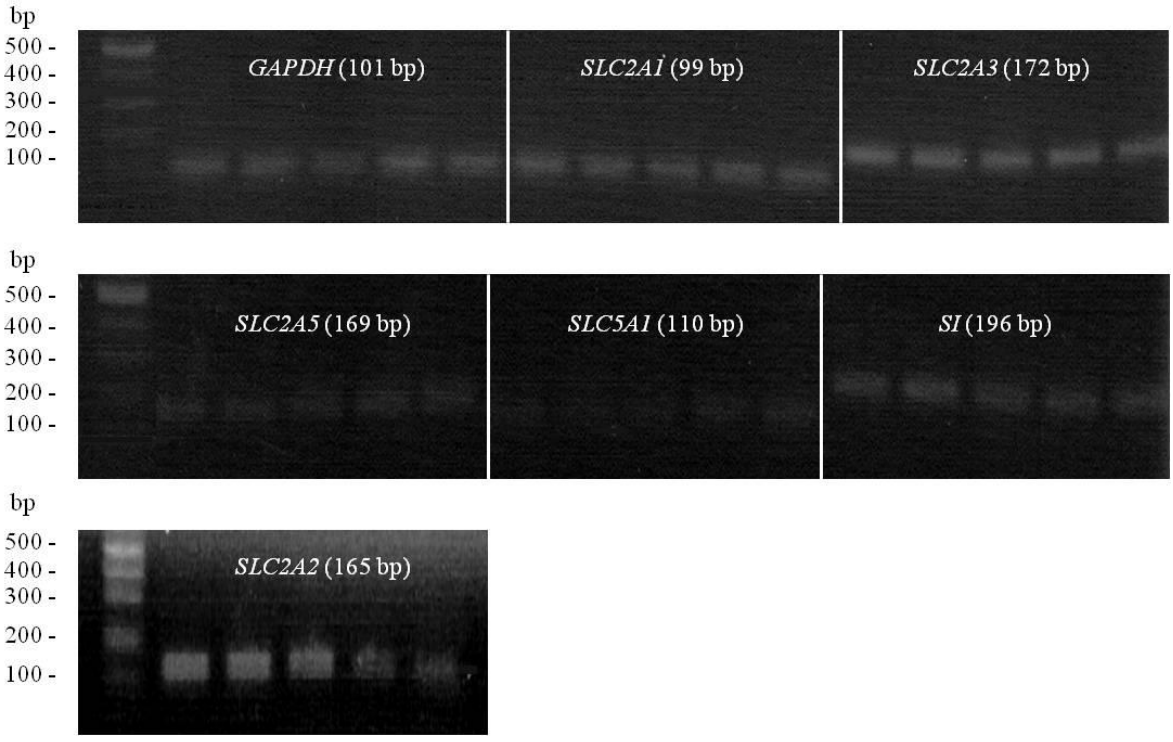

Figure 47. DNA agarose gel electrophoresis images of selected PCR products

Images shown are DNA agarose gel electrophoresis photos of the PCR products of seven genes (named in the figure). Numbers in brackets indicate the predicted PCR amplicon sizes.